REMARKS/ARGUMENTS

I. Status of the Claims

After entry of this amendment, claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-55, 65-68, 70-77, 79-86 and 87-106 are pending. Claims 22-30 are withdrawn.

II. The Invention

It is the discovery of the present inventors that fucosyltransferases can be used *in vitro* to modify glycosylation patterns of glycopeptides. Accordingly the examined claims are drawn to *in vitro* methods for modifying glycosylation patterns of glycopeptides, including recombinantly produced glycopeptides using fucosyltransferases, especially fucosyltransferases that lack their membrane anchoring domain.

III. Arguments for the Patentability of the Pending Claims

The following arguments address issues and/or references cited by the Examiner in previous Office Actions. Applicants respectfully request that the Examiner considers these arguments before the issuance of the next Action in this case. A discussion of the merits of these arguments in the next Action is also respectfully requested.

a) The Examiner has not adequately addressed Applicants' 'Teaching Away' arguments

The MPEP discloses the Examiner's burden upon presentation of 'teaching away' arguments:

> The test for obviousness is what the combined teachings of the references would have suggested to one of ordinary skill in the art, and ***all teachings in the prior art must be considered*** to the extent that they are in analogous arts. ***Where the teachings of two or more prior art references conflict, the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art,*** considering the degree to which one reference might accurately discredit another. *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1989)
>
> MPEP § 2143.01 (emphasis added)

"Proceeding contrary to the accepted wisdom...is 'strong evidence of unobviousness.'" *Ruiz v. Foundation Anchoring Systems, Inc.*, 234 F.3d 654, 667 (Fed.Cir. 2000) (citations omitted). When the teachings of the references conflict, the merits of each reference must be weighed in order to decide which would be most persuasive to one of skill in the art.

During the course of prosecution, Applicants have demonstrated that two cited references 'teach away' from the Applicants invention. These cited references are Costa and Natsuka. Applicants demonstrated that both references teach that removing a transmembrane domain from a eukaryotic fucosyltransferase destroys its ability to fucosylate a glycopeptide, thus preventing it from producing a substantially uniform fucosylation pattern on the glycopeptide. Applicants additionally demonstrated that Costa was more relevant to Applicants' invention than the prior art. The text of both of Applicants' teaching away arguments is attached as **Exhibit 1**.

The Examiner's comments in the Office Action dated March 14, 2005 are non-responsive to Applicants' "teaching away" arguments regarding the references. In this Office Action, the Examiner did not address or refute Applicants' arguments drawn to the two references. Rather, the Examiner repeated a statement from a previous Office Action.

> However, as stated here and in previous Office actions, and reiterated here it is also well known that transferases which are normally membrane bound and have solubility problems and therefore are not suitable for process involving immobilization etc. It is also well known in the art that such problems can be solved by removing membrane spanning domains which render the enzyme

> soluble and thereby increases the efficiency of the enzymatic reaction.
>
> Office Action dated March 14, 2005, page 6

Applicants respectfully submit that the Examiner's assertions run counter to the disclosures of Costa et al., and Natsuka et al., both of which disclose that fucosyltransferases lacking the membrane anchoring domain are unable to fucosylate a glycopeptide, thus preventing it from producing a substantially uniform fucosylation pattern on the glycopeptide. There is currently no art of record that teaches the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. One skilled in the art knowing the disclosure by Costa et al., and Natsuka et al., would have had serious doubts as to whether members of the fucosyltransferase family can be used for producing a substantially uniform fucosylation pattern without their membrane anchoring domain. Even if one skilled in the art would have considered deleting the membrane anchoring domain of a fucosyltransferase, other than FucT-III or FucT-VII, he or she would not have had any reasonable expectation of success of producing a substantially uniform fucosylation pattern in light of the fact that both FucT-III and FucT-VII, according to the prior art disclosure, cannot be used for fucosylation without the membrane anchoring domain.

In order to rebut the Applicants' specific examples, the Examiner should specifically discuss the teachings of Costa and Natsuka in light of the specific cited references. In the absence of such evidence, Applicants' 'teaching away' arguments remain unchallenged.

b) The Methods of the '320 Application and Thomas are substantially the same.

In the Response to the Office Action dated July 9, 2004, Applicants presented a Declaration from Dr. David Zopf, the Executive Vice-President and Chief Scientific Officer of the assignee of U.S. Pat. App. No. 09/855,320 ("the '320 Application"). In paragraph 2 of this Declaration, Dr. Zopf disclosed that the *in vitro* fucosylation methods taught by the '320 Application have enjoyed commercial success. In paragraph 4 of the Declaration, Dr. Zopf disclosed that experts were initially skeptical of *in vitro* glycosylation. Evidence of this skepticism was disclosed in a letter written by Dr. James Bailey that accompanied Dr. Zopf's declaration as Exhibit E. In paragraph 8 of this Declaration, Dr. Zopf described a collaboration with Avant Immunotherapeutics as an example of this commercial success. The results of this

collaboration were published as Thomas, L.J. *et al.*, *Glycobiology* **14(10)**: 883-893 (2004) ("Thomas"). This scientific publication was attached to Dr. Zopf's Declaration as Exhibit B. As Dr. Zopf described in paragraphs 2 and 8 of his Declaration, the *in vitro* fucosylation methods of the '320 Application were used successfully in Thomas.

In the Office Action dated March 14, 2005, the Examiner stated that it is not crystal clear that Thomas employed the *in vitro* fucosylation methods of the '320 Application. As further evidence of this connection between Thomas and the '320 Application, Applicants provide a declaration by Dr. Robert Bayer, an inventor of the '320 Application. Dr. Bayer demonstrates in this declaration, attached as **Exhibit 2**, that the methods and results of the '320 Application are substantially the same as those disclosed in Thomas. As the link between the two documents has been made clear by Dr. Bayer's declaration, Applicants respectfully request that the Examiner fully consider all of the arguments put forward in Dr. Zopf's declaration, including the evidence of expert skepticism provided by the letter of Dr. James Bailey and the examples of commercial success as shown by the collaboration between the assignee of the '320 Application and Wyeth/Ayerst Laboratories, and between the assignee of the '320 Application and Avant Immunotherapeutics.

IV. The Response to the Rejection

a) Under 35 U.S.C. § 103(a)

In order to establish a *prima facie* case of obviousness, the Examiner must demonstrate that (1) the references teach all the claimed elements; (2) there is a suggestion or motivation in the prior art to modify or combine the reference teachings; and (3) there is a reasonable expectation of success. MPEP § 2143; *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991).

Over Paulson, deVries or Malissard and Seed

Claims 1-4, 6, 8, 10-17, 19-21, 31-36, 38, 40, 42-49, 51-52, 66-68, 70-77, and 79-86 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Paulson, *et al.*, (PCT Publication No. 98/31826) ("Paulson"), deVries *et al.* (*J. Biol. Chem.*, **1995**, Vol. 270 (15:8712-

8722)) ("deVries") or Malissard (*BBRC,* **2000**, Vol. 267 (169-173)) ("Malissard") and Seed, *et al.*, (PCT Publication No. 96/40881) ("Seed").

Claims 54-55 and 87-106 are also rejected under 35 U.S.C. § 103(a) as allegedly being obvious over the same combination of references provided above.

Since both rejections rely on the same prior art references, Applicants will address these rejections together.

As explained below, the cited references fail to disclose or suggest Applicants' element of "conditions appropriate . . . such that the glycopeptide has a substantially uniform fucosylation pattern". Since Paulson, deVries, Malissard, and Seed do not contain this element, there is neither a motivation to combine the reference teachings to produce Applicants' invention, nor can a reasonable expectation of success be derived from the references. Therefore, a *prima facie* case of obviousness cannot be made.

(1) The cited references all fail to teach one of the claimed elements

Each of the cited references, Paulson, deVries, Malissard and Seed, fail to teach Applicants' claimed element of "conditions appropriate . . . such that the glycopeptide has a substantially uniform fucosylation pattern".

Paulson is cited by the Examiner for "essentially teach[ing] a method of glycosylation involving an enzyme other than a fucosyltransferase". The Examiner's characterization implies that Paulson teaches a general method of glycosylation. Applicants respectfully disagree. In fact, Paulson's teachings are restricted to the specific use of sialyltransferases for the addition of sialic acid moieties to acceptor moieties. Paulson's disclosure does not mention fucosyltransferase or methods of adding fucose to a glycoprotein. Paulson's disclosure also fails to suggest that his sialylation conditions could be used for any other saccharide or glycosyltransferase. Paulson's methods are discussed specifically within the context of sialylation or their use with sialyltransferases. Since Paulson does not extend his teachings beyond sialylation, it is improper to characterize Paulson as "essentially teach[ing] a method of glycosylation involving an enzyme other than a fucosyltransferase". Rather, Paulson teaches a method of sialylation involving sialyltransferases.

Since Paulson does not explicitly teach the applicability of the disclosed sialylation methods for fucosylation, Paulson can not teach a glycopeptide with a substantially uniform fucosylation pattern. Since Paulson also limits his teachings to only those involving sialylation, Paulson does not suggest or imply that the reaction conditions could be used to produce a glycopeptide with a substantially uniform fucosylation pattern. Therefore, Paulson does not teach Applicants' element of the "conditions appropriate . . . such that the glycopeptide has a substantially uniform fucosylation pattern".

deVries is cited by the Examiner for teaching a process of making soluble fucosyltransferases which lack their membrane anchoring domain. While deVries teaches this process, deVries does not teach a substantially uniform fucosylation pattern on a glycopeptide. On p. 8714, column 1, deVries discloses the fucosylation assay conditions. The amount of fucose donor is reported as 5 nanomoles while the amount of fucose acceptor is reported as 110 nanomoles. Even if 100% of the fucose donors were transferred to the fucose acceptor, still only 4.6% of the fucose acceptor sites would by fucosylated. Since the maximum achievable fucose incorporation rate taught by deVries is 4.6%, and at least 60% is required by the claim element, deVries does not teach Applicants' element of the "conditions appropriate . . . such that the glycopeptide has a substantially uniform fucosylation pattern".

Malissard is also cited by the Examiner for teaching a process of making soluble fucosyltransferases which lack their membrane anchoring domain. While Malissard teaches this process, Malissard neither teaches a fucosylated glycopeptide nor a substantially uniform fucosylation pattern on any glycopeptide. Malissard states that the conditions used were described in Borsig *et al.*, *Glycobiology*, **8**: 259-268 (1998) ("Borsig"). On p. 267, column 2, Borsig discloses the fucosylation assay conditions. The fucose acceptors were α2,3-sialyllactosamine or 2-fucosyllactose, N-acetyllactosamine, and lacto-N-biose I. Since all of these fucose acceptors are oligosaccharides, Malissard (through Borsig) does not teach a fucosylated glycopeptide.

Malissard also does not teach the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. The amount of fucose donor (GDP-fucose) is

reported as 101 micromoles while the amount of fucose acceptor is reported as 5 millimoles. Even if 100% of the fucose donors were transferred to the fucose acceptor, still only 2.0% of the fucose acceptor sites would be fucosylated. Since the maximum achievable fucose incorporation rate taught by Malissard is 2.0%, and at least 60% is required by the claim element, Malissard does not teach Applicant's element of the "conditions appropriate . . . such that the glycopeptide has a substantially uniform fucosylation pattern".

Seed is cited by the Examiner for teaching background information regarding fucosyltransferases and their ability to fucosylate glycopeptides and glycolipids. While Seed teaches this background information, Seed does not teach the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. Since none of the other references teach these conditions, none of the cited references teach or suggest how to produce a glycopeptide with a substantially uniform fucosylation pattern.

Seed is a PCT publication which claims priority to U.S. Pat. App. No. 09/483,151 (now U.S. Pat. No. 5,858,752). The claim set for the PCT publication is listed on pages 41 and 42. Claim 30 is directed to an *in vitro* method of fucosylating a polypeptide by contacting a polypeptide with said fucosyltransferase under conditions sufficient for fucosylating said polypeptide. However there is no discussion, and therefore no enabling disclosure, in Seed of the conditions necessary for producing a glycopeptide with a substantially uniform fucosylation pattern. The fact that U.S. Pat. No. 5,858,752 issued without claim 30 is further evidence of the lack of this enabling disclosure in Seed. Therefore, Seed does not teach the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern.

As discussed above, none of the cited references (Paulson, deVries, Malissard, Seed) teach the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. Paulson does not teach these conditions because the disclosure is restricted to methods of sialylation involving sialyltransferases. deVries does not teach these conditions since deVries' conditions only produce a fucose incorporation percentage of 4.6%. Malissard does not teach these conditions since Malissard's conditions only produce a fucose incorporation percentage of 2%. Seed does not teach these conditions since *in vitro* fucosylation conditions are

not disclosed in the reference. Therefore, none of these references contain the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. Therefore, not all the elements of Applicants' claims are present in the prior art, and an obviousness rejection is improper.

(2) There is no suggestion or motivation to modify the reference teachings

The cited references also fail to suggest the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. As mentioned above, none of the cited references teach or suggest this element of Applicants' invention. Since Paulson, deVries, Malissard and Seed do not contain this element of Applicants' invention, a suggestion or motivation to modify the cited references for the purpose of Applicants' invention is not present. Therefore, a *prima facie* obviousness rejection cannot be maintained.

(3) The cited references do not provide a reasonable expectation of success

The cited references also fail to provide a reasonable expectation of success in performing the Applicants' invention. As mentioned earlier, there is nothing in Paulson, deVries, Malissard, or Seed that discloses or suggests the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern. Since the claimed invention provides the conditions necessary to produce a glycopeptide with a substantially uniform fucosylation pattern, and none of the cited reference teach conditions for producing such a pattern, the cited references do not create a reasonable expectation that their compounds can be successfully used for the purpose of Applicants' invention. Therefore, the *prima facie* obviousness rejection cannot be maintained.

Because the cited references fail to teach all the claimed elements, do not contain a suggestion or motivation to modify the reference teachings, and do not provide a reasonable expectation of success, a *prima facie* case of obviousness cannot be set forth. Thus, Applicants respectfully request withdrawal of the rejection.

b) Numerous Dependent Claims include elements not found in the cited references

The following pending claims all contain elements which are not present in the art currently of record. Therefore, Applicants respectfully request for notification of the allowability of the following claims.

Claim 2 is directed to a method of modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase.

Claim 3 is directed to a method of modifying the glycosylation pattern of a glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase.

Claim 4 is directed to a method of modifying the glycosylation pattern of a glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide without product isolation after the first contacting. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase without product isolation after the first contacting.

Claim 6 is directed to a method of modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof.

Claim 20 is directed to a method further comprising glycosylating a glycopeptide with a glycosyltransferase other than a fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and a glycosyltransferase that is not a fucosyltransferase.

Claim 21 is directed to a method further comprising glycosylating a glycopeptide with a glycosyltransferase that is a galactosyltransferase, sialyltransferase, or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and galactosyltransferase, sialyltransferase, or a combination thereof.

Claim 34 is directed to a method of producing a recombinant glycopeptide which involves a first and second fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase.

Claim 35 is directed to a method of producing a recombinant glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase.

Claim 36 is directed to a method of producing a recombinant glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase.

Claim 38 is directed to a method of fucosylating a glycopeptide in which the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof.

Claim 52 is directed to a method of producing a recombinant glycopeptide further comprising glycosylating the glycopeptide with a glycosyltransferase other than a fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and a glycosyltransferase that is not a fucosyltransferase.

Claim 53 is directed to a method of producing a recombinant glycopeptide further comprising glycosylating the glycopeptide with a glycosyltransferase that is a galactosyltransferase, sialyltransferase, or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and galactosyltransferase, sialyltransferase, or a combination thereof.

Claims 54 and 55 are directed to large-scale methods for modifying the glycosylation pattern of a glycopeptide wherein at least 500 mg of glycopeptide is in the reaction mixture. There is no teaching in the cited art involving large-scale fucosylation of the sort described in the above claims.

Claim 66 is directed to a method of modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase.

Claim 67 is directed to a method of modifying the glycosylation pattern of a glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase.

Claim 68 is directed to a method of modifying the glycosylation pattern of a glycopeptide in which the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof.

Claim 75 is directed to a method of producing a recombinant glycopeptide which involves a first and second fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase. Also, there is no teaching in the cited art of the termination of the transfer of the fucose to the fucose acceptor once the substantially identical fucosylation pattern is obtained.

Claim 76 is directed to a method of producing a recombinant glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase. Also, there is no teaching in the cited art of the termination of the transfer of the fucose to the fucose acceptor once the substantially identical fucosylation pattern is obtained.

Claim 77 is directed to a method of producing a recombinant glycopeptide which involves a second fucosyltransferase which is selected from FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a second fucosyltransferase which is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. Also, there is no teaching in the cited art of the termination of the transfer of the fucose to the fucose acceptor once the substantially identical fucosylation pattern is obtained.

Claim 82 is directed to a method of producing a recombinant glycopeptide further comprising glycosylating the glycopeptide with a glycosyltransferase that is a galactosyltransferase, sialyltransferase, or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and galactosyltransferase, sialyltransferase, or a combination thereof. Also, there is no teaching in the cited art of the termination of the transfer of the fucose to the fucose acceptor once the substantially identical fucosylation pattern is obtained.

Claim 87 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase. Also, there is no teaching in the cited art involving large-scale fucosylation of the sort described in the above claims.

Claim 88 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide simultaneously. In addition to the missing elements described in part a), there is no

teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase. Also, there is no teaching in the cited art involving large-scale fucosylation of the sort described in the above claims.

Claim 89 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide wherein the first and second fucosyltransferase contact the glycopeptide without product isolation after the first contacting. In addition to the missing elements described in part a), there is no teaching in the cited art of a method involving the simultaneous use of a first and second fucosyltransferase without product isolation after the first contacting. Also, there is no teaching in the cited art involving large-scale fucosylation of the sort described in the above claims.

Claim 90 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase wherein the first fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase wherein the first fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof.

Claim 91 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide which involves a first and second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art of a method combining a first and a second fucosyltransferase wherein the second fucosyltransferase is FucT-IV, FucT-V, FucT-VI, FucT-VII or a combination thereof.

Claim 105 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide further comprising glycosylating the glycopeptide with a glycosyltransferase other than a fucosyltransferase. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and a glycosyltransferase that is not a fucosyltransferase.

Claim 106 is directed to a large-scale method for modifying the glycosylation pattern of a glycopeptide further comprising glycosylating the glycopeptide with a glycosyltransferase that is a galactosyltransferase, sialyltransferase, or a combination thereof. In addition to the missing elements described in part a), there is no teaching in the cited art combining a fucosyltransferase and galactosyltransferase, sialyltransferase, or a combination thereof.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

Applicants respectfully request a telephone interview if the Examiner believes that the claims as amended are not in condition for allowance in light of the response submitted above. The undersigned can be reached at 415-442-1000.

Respectfully submitted,

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1-SF/7232846.1

Exhibit 1

Applicants' "Teaching Away" Argument from the Response to the Office Action dated September 17, 2003

The MPEP elucidates the test for this element of the *prima facie* obviousness case:

> The test for obviousness is what the combined teachings of the references would have suggested to one of ordinary skill in the art, and ***all teachings in the prior art must be considered*** to the extent that they are in analogous arts. ***Where the teachings of two or more prior art references conflict, the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art,*** considering the degree to which one reference might accurately discredit another. *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1989)
>
> MPEP § 2143.01 (emphasis added)

"Proceeding contrary to the accepted wisdom...is 'strong evidence of unobviousness.'" *Ruiz v. Foundation Anchoring Systems, Inc.*, 234 F.3d 654, 667 (Fed.Cir. 2000)(citations omitted). Therefore, the 'motivation to combine' is taken not just from the cited references, but from the teachings of the references as a whole. When the teachings of the references conflict, the merits of each reference must be weighed in order to decide which would be most persuasive to one of skill in the art.

The final reference cited by the Examiner is Taylor, a published U.S. patent application. Although they are not yet issued, Taylor's claims are drawn to transmembrane segment-free polypeptides having α1,3-fucosyltransferase activity. Although appearing to encompass both eukaryotic and bacterial fucosyltransferases, it is important to note that all of Taylor's working examples are drawn to bacterial fucosyltransferases, which lack transmembrane domains in their natural state. Moreover, Taylor does not discuss any potential changes in enzyme activity which may arise in a eukaryotic fucosyltransferase upon removal of the transmembrane anchoring domain. Therefore, one of skill in the art would surmise from Taylor that transmembrane domain-less eukaryotic fucosyltransferases and bacterial fucosyltransferases will have similar properties.

The teachings of Taylor are **expressly taught away from** by Costa *et al.*, Stable Expression of the Golgi Form and Secretory Variants of Human Fucosyltransferase III from BHK-21 cells, *J. Biol. Chem.*, **272**: 17, 11613-11621 (1997) ("Costa"). Costa describes a series of scientific experiments on human fucosyltransferase III (FucT-III). When this protein contains its transmembrane anchoring domain, it is capable of fucosylating the substrates for Lewis a and Lewis x. In Costa, the transmembrane anchoring domain of FucT-III was removed. Upon removal, the authors reported an unexpected change in the activity of the enzyme. While the modified FucT-III was able to fucosylate Lewis a, it lost its ability to fucosylate the substrate for Lewis x or sialyl Lewis x. This finding is summarized in Costa's abstract:

> The soluble forms of fucosyltransferase III secreted by stably transfected cells may be used for *in vitro* synthesis of the Lewis a

determinant on carbohydrates and glycoproteins, ***whereas Lewis x and sialyl Lewis x structures cannot be synthesized***.

Costa, *J. Biol. Chem.*, **272**:17, 11613 (abstract) (1997).

Emphasis added.

Costa, therefore, teaches that removal of the transmembrane domain can unpredictably alter the fucosyltransferase activity.

Since the teachings of Costa directly conflict with those of Taylor, the power of the two references to one of skill in the art must be compared. MPEP § 2143.01. Costa is a scientific publication that details the unexpected results of a series of real-world experiments conducted on a eukaryotic fucosyltransferase. On the other hand, Taylor is a published patent application that does not disclose the manipulation of any eukaryotic fucosyltransferases. Since Costa's findings are based on actual experimentation with eukaryotic fucosyltransferases, while Taylor's findings do not, one of skill in the art would find the teachings of Costa to be more powerful and persuasive than the teachings of Taylor.

The power of Costa's teaching away argument is not overcome by the other references cited by the Examiner, Seed and Paulson. Since Paulson does not describe fucosyltransferases, it also does not disclose the use of transmembrane domain-less eukaryotic fucosyltransferases. While Seed discusses eukaryotic fucosyltransferases, it does not mention transmembrane domains or any circumstances that would necessitate their removal. Therefore, these references do not provide a motivation to remove the transmembrane domain from a eukaryotic fucosyltransferase. Since neither the Paulson nor Seed references are germane to the discussion in earlier paragraphs concerning transmembrane domain-less eukaryotic fucosyltransferases, the power of Costa's teaching away argument remains unmitigated.

Based on the discussion above, Costa expressly teaches away from the use of a transmembrane domain-less eukaryotic fucosyltransferase to fucosylate the substrate for Lewis x or sialyl Lewis x. In spite of these warnings in the references, Applicant succeeded in producing transmembrane domain-less eukaryotic fucosyltransferases which are capable of fucosylating the substrate for Lewis x or sialyl Lewis x. Because a reference teaches away from the Applicant's invention, there is no motivation or suggestion in the references for this feature of Applicant's invention. Because the Applicant presents surprising results that refute teachings in the references, the obviousness rejection is improper and cannot be maintained.

Applicants' "Teaching Away" Argument from the Response to the Office Action dated July 9, 2004

Specifically none of the cited prior art references teach or suggest using fucosyltransferases lacking membrane anchoring domains *in vitro*. The Office Action states that "Natsuka et al. teach eukaryotic FTs that lack membrane anchor domain." Applicants respectfully submit that Natsuka does not teach or suggest fucosyltransferases lacking membrane anchoring domains. To the contrary, Natsuka discloses that fucosyltransferases lacking membrane anchoring domain cannot be expressed or obtained using transformed cell lines, thus no fucosylation activity can be detected with fucosyltransferases lacking membrane anchoring domain.

In particular, Natsuka discloses five (5) representative cDNA clones of fucosyltransferase VII, *i.e.* cDNA3, cDNA5, cDNA6, cDNA10, and cDNA14 (Figure 1a, and column 24, lines 45-50). Of these 5 classes of cDNA clones, four of them (cDNA 5, 6, 10, and 14) are trans-membrane proteins while one of them, *i.e.,* cDNA3 lacks the membrane anchoring domain (column 24, lines 58-67, column 25, lines 1-23). Natsuka discloses that "[i]n contrast to the results obtained with cDNAs 5, 6, 10, and 14, cDNA 3 does not direct detectable sLe^x expression." (See column 26, lines 10-11). Natsuka further discloses that "cells transfected with cDNA3 do not contain any detectable immunoreactive proteins" and this suggests that "the putative initiator codon...in this cDNA does not initiate translation of an immunoreactive product", *i.e.,* does not initiate translation of a fucosyltransferase. In essence, Natsuka discloses that it can not obtain a detectable level of FucT-VII lacking the membrane anchoring domain, *i.e.,* the sequence of FucT-VII does not naturally encode a FucT-VII that lacks the membrane anchoring domain. Such results provided by Natsuka would have cast serious doubts as to whether FucT-VII lacking the membrane anchoring domain is obtainable, and if so, whether it would be useful for fucosylation.

In addition to the teaching away by Natsuka's disclosure with respect to FucT-VII, Costa et al., (Journal of Biological Chemistry, vol. 272, No. 17 pp. 11613-11621, 1997) also disclose that another member of fucosyltransferase family, FucT-III, cannot be used to fucosylate glycopeptides after losing its membrane anchoring domain. (See abstract and discussion sections). The Office Action states that because applicants have excluded FucT-III from certain claims, "it cannot be concluded that what happened with FucT-III would also happen in the case of all other FTs." Applicants respectfully submit that such assertion is without any merit and entirely based on hindsight.

At the time of the present invention, one skilled in the art did not have any knowledge of the present invention. Thus it can not be said that one skilled in the art would have known FucT-III is somewhat unique because of what has been disclosed or claimed in the present invention. In other words, the disclosure of Costa et al., has to be viewed by one skilled in the art without the benefit of the teaching provided by the present invention, *e.g.,* without the benefit of knowing that FucT-III has been excluded from certain claims of the present invention. Therefore, according to the disclosure of Costa et al., one skilled in the art would have had serious doubts as to whether fucosyltransferases lacking the membrane anchoring domain could still maintain their activity in light of the fact that FucT-III lacking the membrane anchoring domain was demonstrated to lose its fucosylation activity.

The Office Action further asserts that irrespective of the disclosure provided by Costa et al., one skilled in the art would have deleted the membrane anchoring domain because of the solubility issue associated with membrane bound proteins. Applicants respectfully submit that such assertion is not supported by any evidence and runs directly against the disclosures of Costa et al., and Natsuka et al., both of which disclose that fucosyltransferases lacking the membrane anchoring domain do not have fucosylation activity. In the absence of any teaching or suggestion, one skilled in the art knowing the disclosure by Costa et al., and Natsuka et al., would have had serious doubts as to whether members of fucosyltransferase family can be used for fucosylation without their membrane anchoring domain. Even if one skilled in the art would have considered deleting the membrane anchoring domain of a fucosyltransferase, other than FucT-III or FucT-VII, he or she would not have had any reasonable expectation of success in light of the fact that both FucT-III and FucT-VII, according to the prior art disclosure, cannot be used for fucosylation without the membrane anchoring domain.

Seed discloses the DNA and amino acid sequences of α(1,3)fucosyltransferase and the use of such full length fucosyltransferase for fucosylation of therapeutic peptides. Seed does not teach or suggest that such fucosyltransferase can be used without its membrane anchoring domain for fucosylation of glycopeptides.

Kashem discloses the use of fucosyltransferase for a single fucosylation site on a simple chemical compound, *i.e.,* oligosaccharides. Kashem does not teach or suggest that fucosyltransferases can be used for any fucosylation of chemical compounds without their membrane anchoring domain.

Paulson discloses the use of sialyltransferases for *in vitro* sialylation of glycoproteins. Paulson does not teach or suggest that fucosyltransferases can be used without their membrane anchoring domain for the purpose of fucosylation of glycopeptides. As acknowledged by the Office Action, Paulson is cited to show glycosylation of peptides using enzymes other than fucosyltransferases which is in response to claim limitations such as glycosylation by an enzyme other than an fucosyltransferase.

Therefore, Seed, Kashem, and Paulson can not cure the deficiency of Natsuka.

In summary, none of the cited prior art references teach or suggest the use of fucosyltransferases without their membrane anchoring domain and it would not have been obvious to one skilled in the art that fucosyltransferases without their membrane anchoring domain could be used *in vitro*, especially in light of substantial "teaching away" disclosures in the field. Thus the *in vitro* methods of using fucosyltransferases without their membrane anchoring domain as provided by the present invention are not obvious over the cited prior art.

Exhibit 2

PATENT

Attorney Docket No.: 040853-01-5108-US
Client Ref. No.: NEO00073

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation Number: 1113
Robert Bayer	Examiner: Rao, Manjunath
Application No.: 09/855,320	Technology Center/Art Unit: 1652
Filed: May 14, 2001	DECLARATION OF DR. ROBERT BAYER UNDER 37 C.F.R. § 1.132
For: IN VITRO MODIFICATION OF GLYCOSYLATION PATTERNS OF RECOMBINANT GLYCOPEPTIDES	"THOMAS DECLARATION"
Customer No.: 43850	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Robert Bayer, Ph.D. declare as follows:

1. I am Senior Director of Research at Neose Technologies, Inc. My duties include directing the research operations of approximately 20 scientists at Neose's San Diego facility. Among these research operations are Neose's GlycoAdvance collaborations. GlycoAdvance is the name of our *in vitro* glycosylation technologies. I have over 14 years experience in this technology area. My *Curriculum Vitae* is attached as Exhibit 2A.

2. I am an inventor of the subject matter claimed in U.S. Patent Application No. 09/855,320 entitled "*In Vitro* Modification of Glycosylation Patterns of Recombinant Glycopeptides" ("the '320 Application"). I am familiar with the material contained in this application.

3. The '320 Application discloses glycopeptides with a "substantially uniform glycosylation pattern" prepared through contacting a glycopeptide having a glycosyl acceptor with a glycosyltransferase and a glycosyl donor moiety. The '320 Application further discloses and also claims a glycopeptide with a "substantially uniform fucosylation pattern" prepared by contacting a glycopeptide having a fucosyl acceptor with a fucosyltransferase and a fucosyl donor moiety.

4. In an earlier filed Declaration by Dr. David Zopf ("Zopf Declaration"), a scientific paper, Thomas, L.J. *et al.*, *Glycobiology* **14(10)**: 883-893 (2004) ("Thomas"), was presented. Thomas was the result of a collaboration between the assignee of the '320 Application and Avant Immunotherapeutics, and is attached as Exhibit 2B.

5. I am submitting this declaration to clarify the substantial identity between the methods and results of the '320 Application and those of the Thomas reference.

6. The starting material in an example of the '320 Application and in Thomas were substantially identical. See paragraph 13.

7. The starting material in an example of the '320 Application and in Thomas were submitted to substantially identical fucosylation conditions. The only difference between the examples disclosed in the two documents is the ratio of fucosyl donor to fucosyl acceptor substrate. In the '320 Application, this ratio is 14:1 (donor:acceptor); in Thomas, this ratio is 7:1 (donor:acceptor). See paragraph 14.

8. Because substantially the same starting materials were submitted to substantially the same fucosylation conditions, one of skill in the art would appreciate that the products of the method of the '320 Application and those of Thomas are substantially the same.

9. The '320 Application claims a composition comprising a glycopeptide having a "substantially uniform fucosylation pattern."

10. The term "substantially uniform glycosylation pattern" is defined on page 15, lines 1-3 and 12-15 of the '320 Application as follows:

> A "substantially uniform glycoform" or a "substantially uniform glycosylation pattern," when referring to a glycopeptide species, refers to the percentage of acceptor moieties that are glycosylated by the glycosyltransferase of interest (*e.g.*, fucosyltransferase). . .
>
> The term "substantially" in the above definitions of "substantially uniform" generally means at least about 60%, at least about 70%, at least about 80%, or more preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

According to this definition, a minimum of 60% of the glycosyl acceptor moieties on a glycopeptide must be glycosylated in order for the glycopeptide to possess a "substantially uniform glycosylation pattern". Therefore, for the specific case of fucose, a minimum of 60% of the fucosyl acceptor moieties on a glycopeptide must be fucosylated in order for the glycopeptide to possess a "substantially uniform fucosylation pattern".

11. The 7:1 fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern". Therefore, the products subjected to the 14:1 fucosylation conditions of the '320 Application also possess a "substantially uniform fucosylation pattern". See paragraphs 16-25.

12. To the extent that the fucosylation conditions differ, the fucosylation conditions of the '320 Application would not be expected to reduce the total percentage of fucosylation, because there are more fucose donors present in the reaction mixture.

The starting materials in the '320 Application and in Thomas were substantially identical.

13. In both Thomas and the '320 Application, the starting material was sCR1-S. In both Thomas and the '320 Application, this starting material was produced *in situ* from a sialylation reaction. The similarity in the starting materials was revealed through fluorophore-assisted carbohydrate electrophoresis gel ("FACE gel") analysis. The FACE gel analysis of the

starting material for Thomas was disclosed in lane 3 of Figure 1. The FACE gel analysis of the starting material for the '320 Application was disclosed in the 'sialylated' lane of Figure 3. The band patterns in both lane 3 and the 'sialylated' lane were the same. The higher of the two bands was a monosialylated glycan product (DP = 7) and the lower of the two bands was a disialylated glycan product (DP = 6.2). The structures of these glycan products are attached as part of Exhibit 2C. The monosialylated glycan product is structure A in Exhibit 2C while the disialylated glycan product is structure B of Exhibit 2C.

The same starting materials were subjected to substantially identical fucosylation conditions in the methods of Thomas and in the methods of the '320 Application.

14. Substantially identical fucosylation conditions are disclosed in Thomas and the '320 Application. These conditions are attached as Exhibit 2D. Thomas and the '320 Application disclose that the reaction temperatures as well as concentrations of fucose acceptors (sCR1-S), fucose donors (GDP-fucose) and fucosyltransferases (FT-VI) are the same. In addition, the ratio of fucosyltransferase to fucose acceptor (FT-VI : sCR1-S) is the same for Thomas (0.02 U FT-VI/mg sCR1-S) as for the '320 Application (0.02 U FT-VI/mg sCR1-S). The main difference between the two reaction conditions lies in the ratio of fucose donor to fucose acceptor. For Thomas, this ratio is 7:1. For the '320 Application, this ratio is 14:1. In other words, the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, if the 7:1 fucose donor : fucose acceptor fucosylation conditions of Thomas produce a "substantially uniform fucosylation pattern", then the 14:1 fucose donor : fucose acceptor fucosylation conditions of the '320 Application will also produce a "substantially uniform fucosylation pattern".

The products in the '320 Application and in Thomas are substantially identical.

15. The similarity in the products in Thomas and the '320 Application were revealed through FACE gel analysis. The FACE gel analysis of the products in Thomas was disclosed in lane 4 of Figure 1. The FACE gel analysis of the products in the '320 Application was disclosed

in the 'sialylated and fucosylated' lane of Figure 3. In both lane 4 and the 'sialylated and fucosylated' lane, a band was not present at DP 6.2, indicating the consumption of the unfucosylated, disialylated starting material. In both lane 4 and the 'sialylated and fucosylated' lane, one band was visible slightly below DP 7, with trace bands at higher DP values. The band contained a difucosylated, disialylated product. This product is structure D in Exhibit 2C.

The 7:1 donor : acceptor fucosylation conditions in Thomas yielded a product with a "substantially uniform fucosylation pattern".

16. The product of the 7:1 donor : acceptor fucosylation conditions in Thomas was subjected to HPLC and MALDI-TOF-MS analysis (Thomas, p. 884, column 1). The HPLC results are presented in paragraph 18. The MALDI-TOF-MS results are used in paragraphs 18-25 to determine whether the glycopeptide products of Thomas possess a "substantially uniform fucosylation pattern."

17. The HPLC analysis yielded monosaccharide content information, which was reported in the "sCR1-S/F" column in Table I of Thomas. According to structure D of Exhibit 2C, one of skill would expect the ratios of glucosamine: galactose: mannose: fucose: sialic acid to be 4: 2: 3: 3: 2. The reported relative amounts of these monosaccharides are 48: 27: 35: 39: 28, which reduces to 4: 2.3: 3: 3.3: 2.3. These experimental values correlate well with expected values.

18. The MALDI-TOF-MS analysis yielded molecular weight information about the fucosylation reaction products, which was reported in Figure 7C of Thomas. This molecular weight information was then converted into product percentages which are reported in Table III of Thomas.

19. Whether a glycopeptide has a "substantially uniform fucosylation pattern" was determined by dividing the total number of fucosylated acceptor sites by the total number of potential fucose acceptor sites. Paragraphs 20-23 detail the percentage of glycans that have one, two, three, or four acceptor sites, and the percentage of the acceptor sites that are fucosylated.

These percentages are then used in paragraph 24 to determine the total percentage of fucosylation, or whether a glycopeptide has a "substantially uniform fucosylation pattern".

20. Calculations for glycans with one acceptor site. The percentage of one-acceptor glycans having no fucose is 0.43 + 0.7 + 3.47 = 4.60. The percentage of one-acceptor glycans having one fucose is 0.95 + 1.15 + 7.07 = 9.17. The total percentage of glycans with one-acceptor glycans is (4.60 + 9.17) = 13.77. Of this number, 9.17/13.77 = 67% are fucosylated.

21. Calculations for glycans with two acceptor sites. The percentage of two-acceptor glycans having no fucose is 1.06. The percentage of two-acceptor glycans having one fucose is 1.28 + 2.93 + 17.26 + 0.13 = 21.60. The percentage of two-acceptor glycans having two fucoses is 0.58 + 6.12 + 51.24 + 0.82 = 58.76. The total percentage of glycans with two-acceptor glycans is (1.06 + 21.60 + 58.76) = 81.42. Of this number, 85.4% are fucosylated, as shown below.

$$\frac{(1.06)\times 0+(1.28+2.93+17.26+0.13)\times 1+(0.58+6.12+51.24+0.82)\times 2}{(81.42\times 2)}=85.4\%$$

22. Calculations for glycans with three acceptor sites. The percentage of three-acceptor glycans having no fucose is 0.16. The percentage of three-acceptor glycans having one fucose is 0.38. The percentage of three-acceptor glycans having two fucoses is 1.51 + 0.75 = 2.26. The percentage of three-acceptor glycans having three fucoses is 0.76. The total percentage of glycans with three-acceptor glycans is 3.55. Of this number, 67% are fucosylated, as shown below.

$$\frac{(0.16)\times 0+(0.38)\times 1+(1.51+0.75)\times 2+(0.75)\times 3}{(3.55\times 3)}=67\%$$

23. Calculations for glycans with four acceptor sites. The percentage of four-acceptor glycans having no fucose is 0.18. The percentage of four-acceptor glycans having one fucose is 0.35. The percentage of four-acceptor glycans having two fucoses is 0. The percentage of four-

acceptor glycans having three fucoses is 0. The total percentage of glycans with four-acceptor glycans is 0. The total percentage of glycans with four-acceptor glycans is 0.53. Of this number, 16% are fucosylated, as shown below.

$$\frac{(0.18)\times 0+(0.35)\times 1+(0)\times 2+(0)\times 3+(0)\times 4}{(0.53\times 4)}=16\%$$

24. Based on the percentages of paragraphs 20-23, the total percentage of fucosylation by Thomas is 83%, as shown below.

$$\frac{(13.77)\times 0.67+(81.42)\times 0.854+(3.55)\times 0.67+(0.53)\times 0.16}{(13.77+81.42+3.55+0.53)}=83\%$$

Since the total percentage of fucosylation is at least 60%, the glycopeptides produced by the methods of Thomas possess a "substantially uniform fucosylation pattern."

25. As mentioned in paragraphs 7 and 14, the fucosylation conditions between Thomas and the '320 Application are the same except that the method of the '320 Application utilizes a 2-fold excess of fucose donor (per fucose acceptor) as compared to Thomas. One of skill in the art would expect that more fucose donors would not reduce the total percentage of fucosylation. Therefore, since the 7:1 donor : acceptor fucosylation conditions of Thomas yield a glycopeptide with a "substantially uniform fucosylation pattern," the 14:1 donor : acceptor fucosylation conditions of the '320 Application also yield a glycopeptide with a "substantially uniform fucosylation pattern".

26. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: _____

_____
Robert Bayer, Ph.D.

1-SF/7233111.1

Exhibit 2A

Robert J. Bayer, Ph.D

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(858) 452-2313 (work)
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RESEARCH AND PROFESSIONAL EXPERIENCE

Neose Technologies, Inc., San Diego, CA **1999-Present**

Senior Director, Research

- Promoted to Senior Director, 2001.
- Recipient of Presidents award, 2002.
- Established West Coast facility of Neose (now 20 employees).
- Responsible for directing science at San Diego facility.
- Responsible for carrying out GlycoAdvance collaborations with partners.
- Responsible for monoclonal antibody glycosylation projects at Neose.
- Responsible for carrying out product optimization for three products in pre-clinical development.
- Inventor or co-inventor on over 15 pending patent applications.

Cytel, Inc., San Diego, CA **1990-1999**

Group Leader, Biochemistry

- Promoted to Senior Scientist in 1993, promoted to Group Leader in 1994.
- Recipient of President's Award in 1994.
- Responsible for molecular biology and biochemistry, including discovery research, cloning, expression, and purification methods development of glycosyltransferases used in manufacturing , and process development and generation of manufacturing directions for enzymatic carbohydrate synthesis.
- Discovered and patented method allowing near 100% yields for enzymatic carbohydrate synthesis, planned and carried out all scale-up from milligram to kilogram scale manufacturing for four products.
- Carried out or supervised the enzymatic steps under cGMP's in the manufacture of Phase I and Phase II clinical supplies of Cylexin.
- Developed or supervised the development of purification protocols for recombinant enzymes used at Cytel for Cylexin™ manufacturing from a variety of expression systems (mammalian, bacterial, fungal, insect cell).
- Developed methods for examining selectin-ligand interactions including ELISA, flow cytometry, fluorescence, and equilibrium dialysis.

As part of a collaborative effort with Abbott Laboratories, developed enzymatic synthesis technology to manufacture a human milk oligosaccharide for inclusion in their nutritional product lines.

- Successfully transferred technology to Abbott CAPD, and assisted in manufacturing multiple kilogram batches on site at Abbott used in human clinical trials.
- Developed enzymatic synthesis technology to produce other carbohydrates with business partners to be used in medical devices as well as consumer products.

- Developed glycoprotein remodeling technology to 'repair' incorrect glycosylation on recombinant glycoprotein therapeutics in collaboration with several other biotechnology companies.
- Developed or supervised development of assays suitable for high throughput screening for inhibitors of glycosyltransferases.
- Planned and carried out all purification process development, manufacturing, analytical development, and stability/formulation testing for CY1748, Cytel's humanized anti-P-selectin monoclonal antibody.

Pharmacia Genetic Engineering **1988-1990**
Research Scientist

Developed purification protocols for recombinant HIV proteins to be used in clinical diagnostics.
- Carried out or supervised microbial fermentation.
- Purification and characterization of recombinant proteins from procaryotic and eukaryotic expression systems; renaturation of proteins from inclusion bodies.
- Synthesis, purification, and conjugation of peptides for use as immunogens.

University of California at San Diego **1984-1988**
Postdoctoral Associate, Chemistry Department, Supervisor Prof. J. Kyte

- Developed a new methodology for determining the topology of membrane proteins, and applied this technology to the sodium-potassium ATPase.

Kyoto University **1983-1984**
Researcher, Department of Biophysics, Supervisor Prof. S.I. Ohnishi

- Investigated production of 2-D crystals of influenza virus hemagglutinin for structural determination.

Cornell University **1979-1983**
Graduate Student, Biochemistry and Mol. Biol., Supervisor Prof. G. Feigenson

- Developed a new reconstitution method for M13 coat protein. This was then used to characterize protein partitioning in model membranes undergoing divalent cation-induced phase changes using ESR and fluorescence spectroscopy.

University of Michigan **1976-1979**
Research Associate, Department of Biochemistry, Supervisor Prof. J. Shafer

- Used deuterium isotope effects, UV-visible difference spectroscopy, CD and NMR spectroscopy, and measurement of steady-state and pre-steady state kinetic parameters to elucidate the catalytic mechanism of D-serine dehydratase, an enzyme that uses pyridoxal phosphate as a cofactor.

Education, Awards, Honors, and Fellowships

Ph.D in Biochemistry, 1983 Cornell University
BS Chem with Distinction, High Honors in Chemistry, University of Michigan (1976)
Phi Lambda Upsilon, 1976
U of M Gomberg Prize in Chemistry, 1975, 1976
U of M Class Honors 1974-1976
U of M College Honors Program 1972-1976
National Merit Finalist, UDHS 1972

Publications

Thomas LJ, Panneerselvam K, Beattie DT, Picard MD, Xu B, Rittershaus CW, Marsh HC Jr, Hammond RA, Qian J, Stevenson T, Zopf D, Bayer RJ. Production of a complement inhibitor possessing sialyl Lewis X moieties by *in vitro* glycosylation technology. Glycobiology. 2004 Oct;14(10):883-93.

Gilbert, M., Bayer, R., Cunningham, A.M., DeFrees, S., Gao, Y.H., Watson, D., Young, N.M., and Wakarchuk, W.W. Construction of a CMP-Neu5Ac synthetase/sialyltransferase fusion protein and its use in the synthesis of sialylated oligosaccharides. Nature Biotechnology, 1998, 16(8):769-72.

Phillips, M.L., Schwartz, B.R., Etzioni, E., Bayer, R., Ochs, H.D., Paulson, J.C., and Harlan, J.M. Neutrophil Adhesion in Leukocyte Adhesion Deficiency Syndrome Type 2 J. Clin. Invest. 96, 2898-2906 (1995)

Mehdi, H., Kaplan, M.J., Anlar, F.Y., Yang, X., Bayer, R., Sutherland, K., and Peeples, M.E. Hepatitis B Virus Surface Antigen Binds to Apolipoprotein H. Journal of Virology 68, 2415-2424 (1994)

Mulligan, M.S., Polley, M.J., Bayer, R., Nunn, M.F., Paulson, J.C., and Ward, P.A. Neutrophil-dependent Acute Lung Injury. J. Clin. Invest. 90, 1600-1607 (1992)

Ichikawa, Y., Lin, Y.C., Dumas, D.P., Shen, G.J., Garcia-Junceda, E., Williams, M.A., Bayer, R., Ketcham, C., Walker, L.E., Paulson, J.C., and Wong, C.H. Chemical-Enzymatic Synthesis and Conformational Analysis of Sialyl Lewis X and Derivatives. J. Am. Chem. Soc. 114, 9283-9298 (1992)

Bayer, R. Topological Disposition of the Sequences -QRKIVE- and -KETYY in Native ($Na^+ + K^+$)-ATPase. Biochemistry 29, 2251-2256 (1990)

Kyte, J., Xu, K.Y., and Bayer, R. Demonstration that Lysine 501 of the Alpha Polypeptide of Native Sodium and Potassium Ion Activated Adenosine Triphosphatase is Located on its Cytoplasmic Surface. Biochemistry 26, 8350-8360 (1987)

Bayer, R. and Feigenson, G.W. Reconstitution of M13 Bacteriophage Coat Protein. A New Strategy to Analyze Configuration of the Protein in the Membrane. Biochim. et Biophys. Acta 815, 269-379 (1985)

Federiuk, C.S., Bayer, R.J., and Shafer, J.A. Characterization of the Catalytic Pathway of D-Serine Dehydratase. J. Biol. Chem. 258, 5379-5386 (1983)

Wilson, J.M., Bayer, R.J., and Hupe, D.J. Structure Reactivity Correlation for the Thiol-Disulfide Interchange Reaction. J. Am. Chem. Soc. 99, 7922-7926 (1977)

Spangler, C.W., Hardy, L.W., and Bayer, R.J. Unusual Product Distributions in the Dehydrobrominations of Dibromomethylcyclohexanes. J. Chem. Soc. D 1971, 1416-1417

Patents

Issued US Patents:

US Patent no. 6,033,667 Method for detecting the presence of P-selectin
Chesnut; Robert W.; Polley; Margaret J.; Paulson; James C.; Jones; S. Tarran; Saldanha; Jose W.; Bendig; Mary M.; Kriegler; Michael; Perez; Carl; **Bayer; Robert;** Nunn; Michael

US Patent No. 5,800,815 Antibodies to P-selectin and their uses
Chestnut; Robert W.; Polley; Margaret J.; Paulson; James C.; Jones; S. Tarran; Saldanha; Jose W.; Bendig; Mary M.; Kriegler; Michael; Perez; Carl ; **Bayer; Robert** ; Nunn; Michael

US Patent No. 5,922,577 Enzymatic synthesis of glycosidic linkages
Defrees; Shawn; **Bayer; Robert J.** ; Ratcliffe; Murray

US Patent No. 5,876,980 Enzymatic synthesis of oligosaccharides
DeFrees; Shawn; **Bayer; Robert J.**; Ratcliffe; Murray

US Patent No. 5,728,554 Improved Enzymatic Synthesis of Glycosidic Linkages
Bayer, R.J., DeFrees, S., & Ratcliffe, M.

US Patent No. 6,399,336 Practical in vitro sialylation of recombinant glycoproteins
Paulson; James C.; **Bayer; Robert J.**; Sjoberg; Eric

US Patent No. 6,030,815 Enzymatic synthesis of oligosaccharides
DeFrees; Shawn; **Bayer; Robert J.;** Ratcliffe; Murray

US Patent Applications

Currently inventor or co-inventor on over 15 pending US patent applications.

Exhibit 2B

Glycobiology vol. 14 no. 10 pp. 883–893, 2004
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Advance Access publication on June 9, 2004

Production of a complement inhibitor possessing sialyl Lewis X moieties by *in vitro* glycosylation technology

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Recombinant soluble human complement receptor type 1 (sCR1) is a highly glycosylated glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. sCR1-sLe^x produced in the FT-VI-expressing mutant CHO cell line LEC11 exists as a heterogeneous mixture of glycoforms, a fraction of which include structures with one or more antennae terminated by the sialyl Lewis X (sLe^x) [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc]) epitope. Such multivalent presentation of sLe^x was shown previously to effectively target sCR1 to activated endothelial cells expressing E-selectin. Here, we describe the use of the soluble, recombinant α2-3 sialyltransferase ST3Gal-III and the α1-3 fucosyltransferase FT-VI *in vitro* to introduce sLe^x moieties onto the N-glycan chains of sCR1 overexpressed in standard CHO cell lines. The product (sCR1-S/F) of these *in vitro* enzymatic glycan remodeling reactions performed at the 10-g scale has approximately 14 N-glycan chains per sCR1 molecule, comprised of biantennary (90%), triantennary (8.5%), and tetraantennary (1.5%) structures, nearly all of whose antennae terminate with sLe^x moieties. sCR1-S/F retained complement inhibitory activity and, in comparison with sCR1- sLe^x produced in the LEC11 cell line, contained twice the number of sLe^x moieties per mole glycoprotein, exhibited a twofold increase in area under the intravenous clearance curve in a rat pharmacokinetic model, and exhibited a 10-fold increase in affinity for E-selectin in an *in vitro* binding assay. These results demonstrate that *in vitro* glycosylation of the sCR1 drug product reduces heterogeneity of the glycan profile, improves pharmacokinetics, and enhances carbohydrate-mediated binding to E-selectin.

Key words: glycoengineering/glycoprotein remodeling/glycosylation/glycosyltransferase

Introduction

Soluble complement receptor type 1 (sCR1) is a recombinant glycoprotein that has been shown to inhibit the progression of the complement cascade in both the classical and alternative pathways by inhibiting the stable formation of C3 and C5 convertases and by serving as a cofactor in the proteolytic degradation of C3b and C4b by Factor I (Weisman *et al.*, 1990). The administration of sCR1 has been shown to be effective in a number of animal disease models of human complement–dependent ischemia-reperfusion injury for tissues, such as heart (Lazar *et al.*, 1999), liver (Lehmann *et al.*, 1998), hind limb (Kyriakides *et al.*, 2001a), lung (Naka *et al.*, 1997), and intestine (Williams *et al.*, 1999). Complement inhibition by sCR1 has been shown to reduce hyperacute rejection (Pruitt *et al.*, 1997) and enhance graft survival in many established transplant models (Kallio *et al.*, 2000; Pratt *et al.*, 1996; Stammberger *et al.*, 2000).

In some clinical situations, complement inhibition therapy could be more effective if it were targeted directly to sites of endothelial activation. At sites of inflammation, activated endothelial cells express E-selectin and P-selectin, surface adhesins with carbohydrate-binding domains that recognize the carbohydrate epitope, sLe^x (Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) (Lasky, 1995).

Previously we have described sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), a variant of the sCR1 glycoprotein conveniently produced in LEC11 cells transfected with the sCR1 gene. LEC11 is a mutant Chinese hamster ovary (CHO) cell line that expresses fucosyltransferase VI (FT-VI), a Golgi enzyme capable of adding fucose in α1-3 linkage to GlcNAc in oligosaccharide chains that terminate with either Galβ1-4GlcNAcβ1 ... or Neu5Acα2-3Galβ1-4GlcNAcβ1 ...(Zhang *et al.*, 1999). Of the 25 potential N-glycosylation sites within the sCR1 polypeptide sequence, 13–15 are occupied, the majority with biantennary chains, creating the possibility for as many as 30 sLe^x moieties per molecule of sCR1-sLe^x. However, a previously reported analysis of the N-glycans of sCR1-sLe^x showed heterologous oligosaccharides with a variety of partially sialylated and fucosylated structures yielding less than the maximal number of sLe^x moieties (Picard *et al.*, 2000; Rittershaus *et al.*, 1999). Similar heterogeneity of glycans in CHO-expressed glycoproteins has been described previously and attributed to incomplete Golgi processing, post-secretion degradation due to glycohydrolases released into cell culture media, or both (Goochee *et al.*, 1991; Jenkins *et al.*, 1996).

In this article we describe a process to introduce sLe^x moieties onto the N-glycan chains of sCR1 produced in standard CHO cell lines using *in vitro* enzymatic synthesis.

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This method employs serial treatment of sCR1 with soluble recombinant rat ST3Gal-III and human FT-VI to give an sCR1-sLe^x product, designated sCR1-S/F (for differentiation from the LEC11 product) in which the antennae of N-glycans are nearly uniformly terminated with sLe^x epitopes. The benefits of *in vitro* glycan remodeling include improved pharmacokinetics, enhanced binding to E-selectin, and a means to improve product homogeneity. Enzymatic remodeling is demonstrated at the 10-g scale.

Results

In vitro *remodeling of sCR1 glycans*

sCR1 (250 mg) expressed in CHO cells was sialyated by treatment with ST3Gal-III plus CMP-sialic acid to give a product designated sCR1-S. After an aliquot was removed from the reaction mixture for analysis, the remaining sCR1-S was fucosylated in the same reaction vessel by the addition of FT-VI plus GDP-fucose to give a product designated sCR1-S/F. After purification by serial chromatography on ceramic hydroxyapatite and Q Sepharose, the reaction products had the same retention time and percent purity (98.5%) by reversed phase high-pressure liquid chromatography (RP-HPLC) as the starting material, sCR1 (data not shown). Chemical and functional properties of these molecules were compared with those of sCR1-sLe^x, a molecule previously produced in the FT-VI-expressing LEC11 CHO cell line and shown to contain some N-linked biantennary glycans terminated with the sLe^x tetrasaccharide (Picard *et al.*, 2000).

From the mannose content of sCR1, sCR1-S, sCR1-S/F, and sCR1-sLe^x (Table I) it may be inferred that these molecules contain ~13–15 N-glycan chains per mol protein (assuming 3 mol mannose per N-glycan chain). The fluorophore-assisted carbohydrate electrophoresis (FACE) oligosaccharide profile for sCR1 (Figure 1) shows three major bands consistent with a biantennary structure containing zero, one, or two sialic acid residues, as described previously (Picard *et al.*, 2000). The monosaccharide composition of sCR1 (Table I) suggests that ~57% of total galactosyl residues are substituted with sialic acid (19 mol sialic acid/ 33.2 mol galactose). By comparison, the FACE oligosaccharide profile for sCR1-S (Figure 1) shows one major band that migrates at a position consistent with a biantennary structure containing two sialic acid residues, and monosaccharide analysis reveals the galactose/sialic acid ratio to be 1:1 (Table I).

FACE analysis of glycans from sCR1-S/F, prepared by enzymatic fucosylation of sCR-S, suggests that N-glycans are predominantly biantennary and that fucosylation at both antennae is nearly complete (Figure 1). The dominant oligosaccharide band derived from sCR1-S/F was cut out and extracted from the gel. Sequential removal of monosaccharide residues from the extracted glycoprotein using specific glycosidases gave products with mobilities consistent with α1-6 core-fucosylated, biantennary N-glycans (Figure 2). Monosaccharide analysis of sCR1-S/F shows the presence of 39.3 moles fucose per mol sCR1-S/F, a figure in agreement with the prediction from theory that 39–45 fucose residues per mol protein would be present if all N-glycans were core fucosylated and enzymatic fucosylation of antennary GlcNAc residues were complete.

The FACE oligosaccharide profile for sCR1-sLe^x, a glycoprotein produced in LEC11 CHO cells, shows at least seven bands (Figure 1) with some common to sCR1 and others shown previously (Picard *et al.*, 2000) to represent core fucosylated structures with α1-3 fucosylation at one or more antennae. Heterogeneity in the degree of fucosylation of the N-glycan chains from sCR1-sLe^x also can be appreciated from the results of monosaccharide analysis (Table I). For example, it may be calculated (assuming 3 mannose residues per chain) that sCR1-sLe^x contains an average of 2.5 fucosyl residues per glycan chain. By contrast, the fucose content per glycan chain increases from 0.95 for

Table I. Monosaccharide Content (mol/mol glycoprotein) by HPLC analysis

	sCR1	sCR1-S	sCR1-S/F	sCR1-sLe^x
Glucosamine	62	48	48	62
Galactose	33	28	27	38
Mannose	44	39	35	40
Fucose	16	12	39	33
Sialic acid	19	30	28	27
Sialic acid/galactose	0.57	1.09	1.06	0.70
Glycosylation sites/sCR1	15	13	12	13
Estimated sLe^x/sCR1-sLe^x*	n.a.	n.a.	28	14

*Estimated sLe^x/sCR1-sLe^x = (Fuc/sCR1-sLe^x − sites/sCR1-sLe^x) × Sial/Gal ratio.

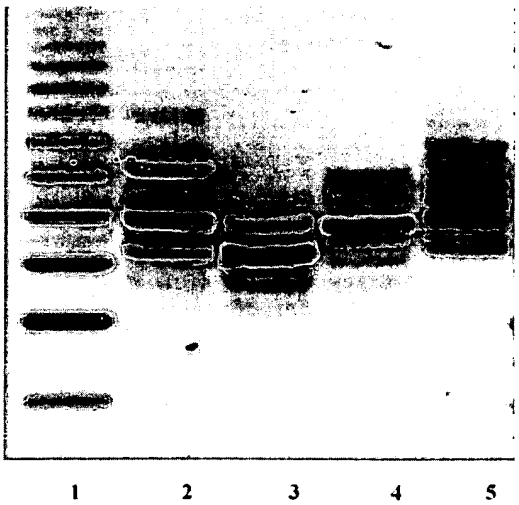


Fig. 1. FACE profiling of oligosaccharides from sCR1-sLe^x and sCR1 before and after enzymatic remodeling: (1) Glyko oligosaccharide standard ladder, (2) sCR1, (3) sCR1-S, (4) sCR1-S/F, (5) sCR1-sLe^x. The oligosaccharide profile of sCR1 (lane 2) contains predominantly bands representing biantennary structures with two sialic acids (bottom band), one sialic acid (middle band), and no sialic acids (top band).

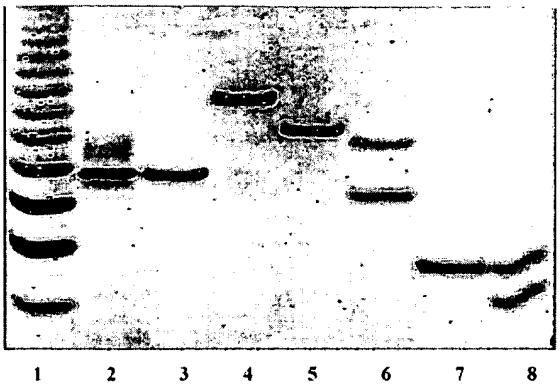


Fig. 2. FACE analysis of oligosaccharides from sCR1-S/F after serial treatment with glycosidases. The dominant oligosaccharide band derived from sCR1-S/F (lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was digested sequentially to remove each monosaccharide residue starting at the terminal sialic acid residue and ending at the trimannosyl core: (1) Glyko oligosaccharide standard ladder; (2) total N-linked oligosaccharides of sCR1-S/F; (3) purified dominant band (band 1) from lane 2; (4) band 1 treated with NANaseIII (cleaves α2-3, 4, 6, 8, and 9 linked sialic acid); (5) band 1 treated with NANaseIII and FucaseIII (cleaves α1-3 and 4 fucose); (6) band 1 treated with NANaseIII, FucaseIII, and GalaseIII (cleaves terminal galactose); (7) band 1 treated with NANaseIII and FucaseIII, GalaseIII, and hexosaminidase; (8) standard trimannosyl core N-glycans with (upper band) and without α1-6 fucose.

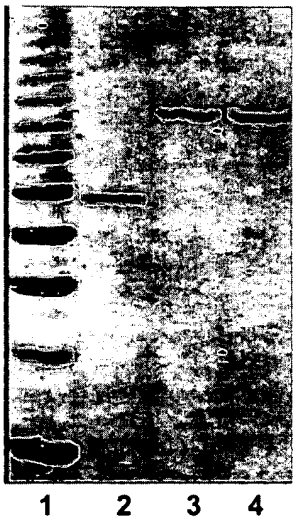


Fig. 3. FACE analysis of oligosaccharides from sCR1-S/F treated with sialidases. The dominant oligosaccharide band (band 1) derived from sCR1-S/F (see Figure 2, lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was subjected to enzymatic digestion to remove terminal sialic acid: (1) Glyko oligosaccharide standard ladder; (2) band 1 from sCR1-S/F; (3) band 1 treated with NANaseI (cleaves α2-3 linked sialic acid); (4) band 1 treated with NANaseIII (cleaves α-3, 4, 6, 8, and 9 linked sialic acid).

sCR1-S to 3.3 for sCR1-S/F, a result that correlates well with the single band visualized by FACE analysis of sCR1-S/F (Figure 1).

Oligosaccharide sequencing using FACE

The linkage of terminal sialic acids on sCR1-S/F was assessed by digestion with specific neuraminidases (Figure 3). Complete removal of sialic acid by treatment of band 1 from sCR1-S/F with NANase I indicates that sialic acid residues are α2-3 linked, as expected.

Optimization of sialylation reaction for scale-up

To establish conditions for scaleup of sialylation, sCR1 (5 mg/ml) was incubated with varying amounts of ST3Gal-III (10, 25, 75, 100, 200, 300 and 400 U/ml) and 5 mM CMP–sialic acid plus a trace amount of radiolabeled CMP–sialic acid for 24 h at 32°C. At an ST3Gal-III concentration of 150 mU/ml, incorporation of radiolabeled sialic acid reached 91% of maximum after 24 h and 100% at 48 h. The lowest concentration of enzyme required to give nearly maximum incorporation (~40 mol sialic acid/mol protein) under these conditions was 25 mU/ml ST3Gal-III (Figure 4). It should be noted that the contribution of triantennary and tetraantennary species may be responsible for the observation that more than 30 moles of sialic acid was added per mole of sCR1. Increasing the CMP–sialic acid concentration from 5 mM to 10 mM did not affect the level of sialylation of sCR1 at any of the ST3Gal-III concentrations tested (data not shown). HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of glycans

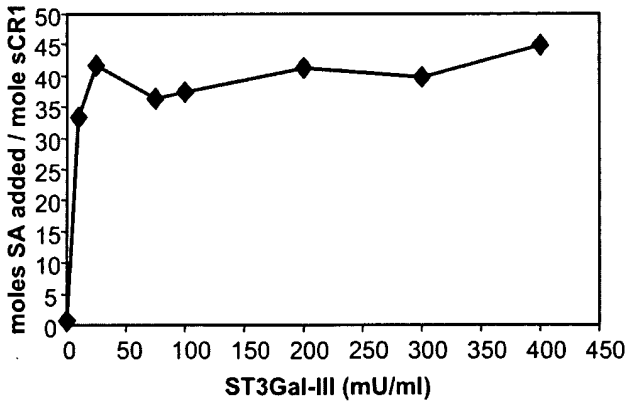


Fig. 4. Incorporation of sialic acid into sCR1 at increasing concentrations of ST3Gal-III in a 24-h reaction. The moles of sialic acid added are estimated from incorporation of radiolabeled CMP–sialic acid. Incorporated radiolabel is separated from free by gel filtration on a $TSKG2000_{SWXL}$ column.

released from sCR1-S revealed that at all concentrations of enzyme tested, the product contained predominantly disialylated, biantennary, core fucosylated N-glycans (data not shown). A concentration of 200 mU ST3Gal-III/ml was chosen for scale-up to ensure completeness of reaction.

Optimization of fucosylation

To establish conditions for scale-up of fucosylation, sCR1-S (5 mg/ml) was incubated with varying amounts of

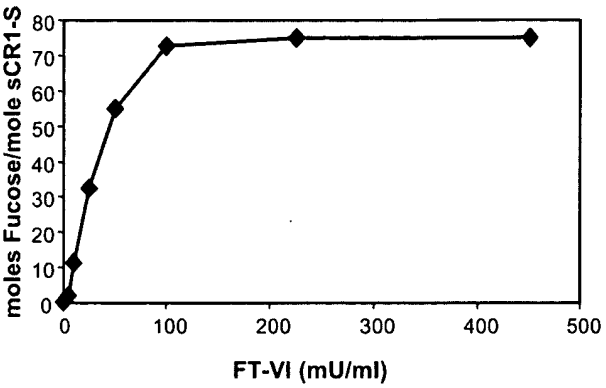


Fig. 5. Incorporation of fucose into sCR1-S at increasing concentrations of FT-VI in a 24-h reaction. The moles of fucose added are estimated from incorporation of radiolabeled GDP-fucose. Incorporated radiolabel is separated from free by gel filtration on a $TSKG2000_{SWXL}$ column.

FT-VI (10, 20, 40, 60, 100, 220, 440 mU/ml) and 5 mM GDP-fucose plus a trace of radiolabeled GDP-fucose for 24 h at 32°C. The lowest concentration of enzyme required to give nearly maximum incorporation of fucose under these conditions was 100 mU/ml FT-VI (Figure 5). Increasing the GDP-fucose concentration from 5 mM to 10 mM did not increase fucose incorporation at several different FT-VI concentrations tested (data not shown).

For products of reactions run at all concentrations of FT-VI $\geq$ 100 mU/ml, the glycan structures identified by HPLC and MALDI-TOF MS were almost the same and essentially indistinguishable from the structures described next for sCR1-S/F produced at the 10-g scale.

Remodeling at 10-g scale

Purified sCR1 (10 g in a volume of 2 L) was incubated first with ST3Gal-III plus CMP–sialic acid at 32°C for 36 h and then, following addition of FT-VI plus GDP-fucose, incubated at 32°C for another 36-h period..

FACE analyses of glycans from sCR1, sCR1-S, and sCR1-S/F for reactions performed at the 10-g scale (data not shown) were essentially indistinguishable from FACE results obtained at the 250-mg scale (Figure 1), suggesting that occupancy of potential acceptor sites for ST3Gal-III and FT-VI on sCR1 at the 10-g scale was nearly complete.

HPLC profiles for 2-AA-derivatized glycans of sCR1, sCR1-S, and sCR1-S/F are shown in Figure 6 and the percentages of glycan species estimated from integrated peak areas are summarized in Table II. After *in vitro* sialylation with ST3Gal-III, neutral glycans, comprising 50% of carbohydrate chains in sCR1, are reduced to 2% of chains in sCR1-S, and monosialo-glycans likewise decrease to from 35% in sCR1 to 17.5% in sCR1-S (Figure 6 and Table II). Overall, about 90% of N-glycans are biantennary and these chains contain an average of 1.8 sialic acid moieties per glycan. Among the minority of biantennary glycans on sCR1-S that are monosialylated, some lack galactose on one antenna, whereas others contain two galactosyl residues, only one of which is sialylated. The remaining 10% of glycans are fully sialylated triantennary (8.5%) or tetra-antennary (1.5%) structures.

After fucosylation of sCR1-S to create sCR1-S/F, HPLC and MALDI-TOF MS analyses (Table III and Figure 7)

Table II. HPLC data summary of large scale remodeling

Glycan species	Native protein (sCR1) (%)	Sialylated protein (sCR1-S) (%)	Sialylated and fucosylated protein (sCR1-S/F) (%)
Neutral	50.5	2.0	4.0
1 charge	35.0	17.5	25.5
2 charges	13.0	70.5	68.5
3 charges	1.5	8.5	1.5
4 charges	ND*	1.5	0.5

*Not detected.

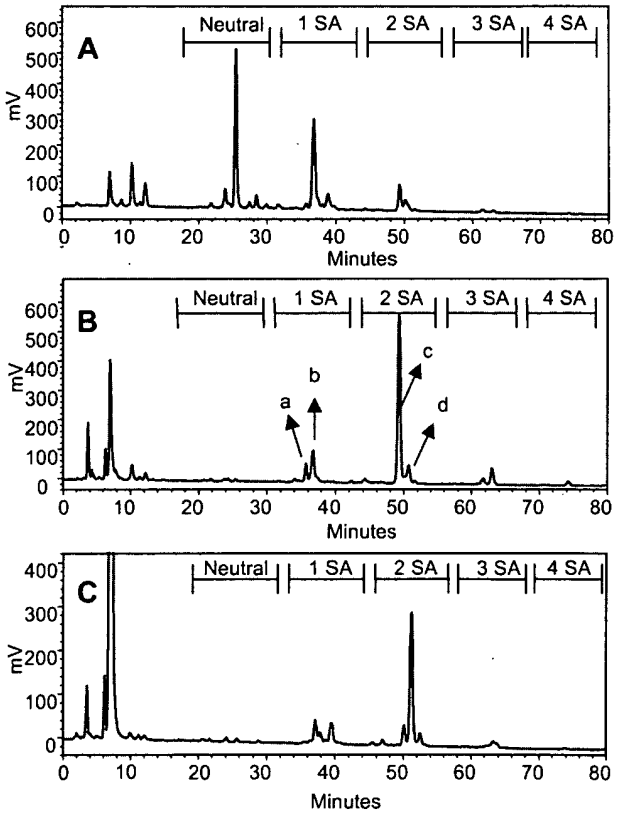
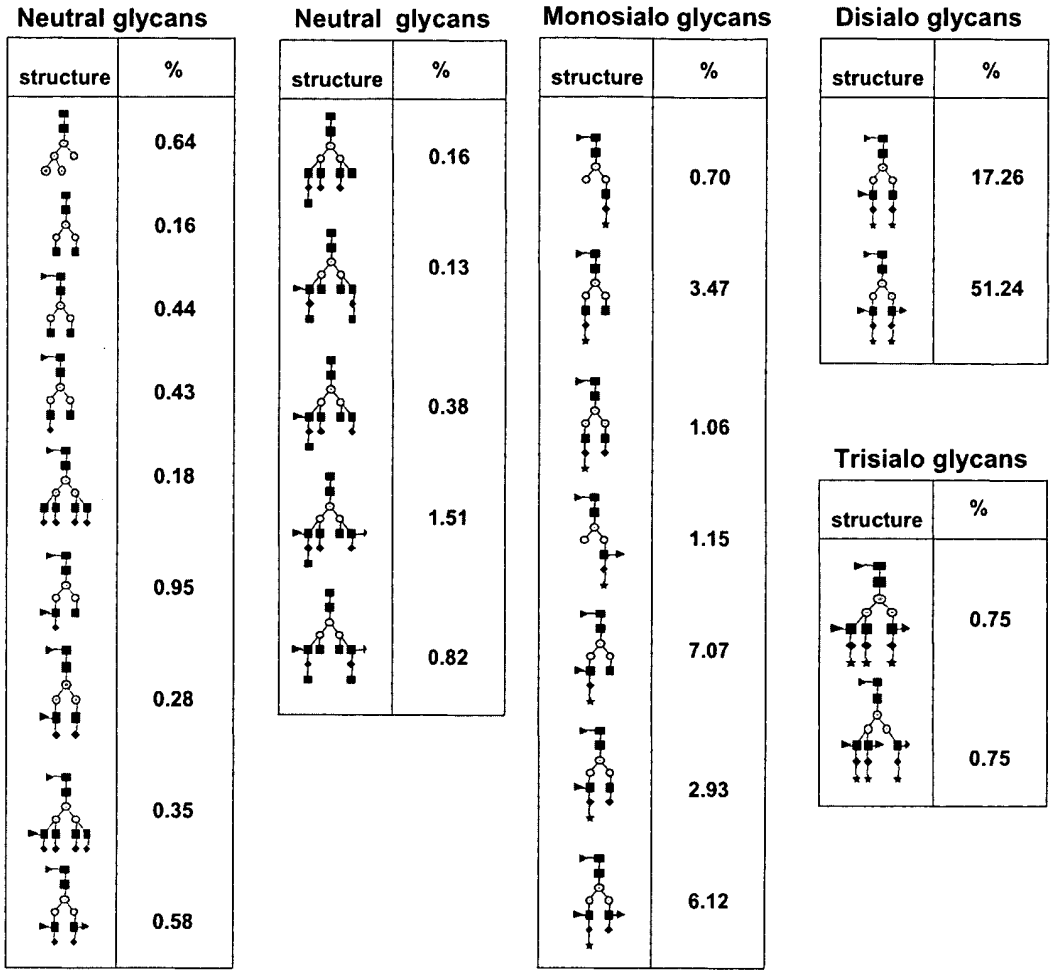


Fig. 6. RP-HPLC analysis of 2-AA-oligosaccharides before and after enzymatic remodeling at the 10-g scale: (**A**) sCR-1; (**B**) sCR1-S; (**C**) sCR1-S/F. MALDI-TOF MS analysis (data not shown) of 2-AA-oligosaccharides from sCR1-S (**B**) indicated that: peak a contains monosialylated biantennary glycans that lack terminal galactose on one antenna; peak b, constituting 12% of biantennary glycans, contains biantennary glycans with two galactose residues, but only one sialic acid; peaks c and d contain disialylated, biantennary glycans, with and without core fucose, respectively.

Table III. sCR1-S/F glycans



Blue squares represent N-acetylglucosamine, yellow circles represent mannose, green triangles represent fucose, red diamonds represent galactose, and asterisks represent sialic acid.

showed that more than 95% of the glycans were fucosylated by FT-VI. About 62% of the total N-glycans gained two fucose residues, and ~30% gained a single fucose residue. Failure to accept two fucosyl residues was in part due to missing galactosyl residues on one or more antennae. From these results it can be estimated that the sCR1-S/F molecules created by consecutive *in vitro* sialylation and fucosylation reactions contain, on average, 28 sLe^x epitopes per protein molecule, whereas sCR1-sLe^x, glycosylated and secreted by the FT-VI-expressing LEC11 CHO cell, contains ~14 sLe^x epitopes per protein molecule (Table I).

To check the stability of sCR1 under conditions of incubation with glycosyltransferases, a small amount of protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after each remodeling reaction. There was no evidence of degradation of the polypeptide after incubation with either ST3Gal-III or FT-VI (data not shown).

Pharmacokinetics

When sCR1-S prepared at the 250-mg scale was injected intravenously into rats, the observed area under the curve (AUC_{last}) was twofold greater than the AUC_{last} for sCR1 ($p < 0.004$), indicating a significantly greater exposure of the more completely sialylated form of the complement inhibitor to intravascular cells following dosing (Figure 8).

In vitro *antihemolytic activity*

The IH_{50} values for sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F as inhibitors of human complement–mediated lysis of sheep red blood cells were found to be similar (Figure 9 and

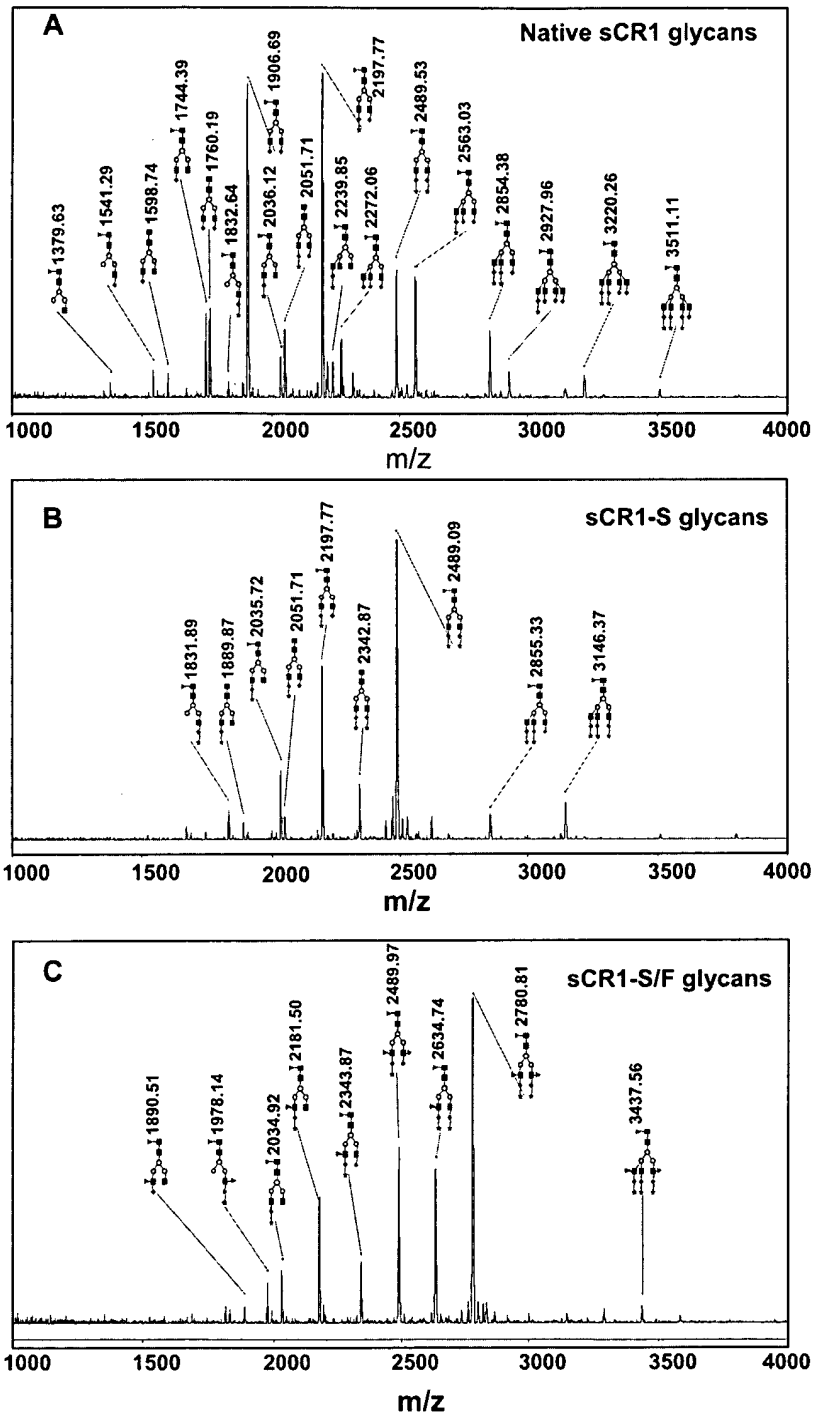


Fig. 7. MALDI-TOF analysis of total glycans from (**A**) sCR1, (**B**) sCR1-S, and (**C**) sCR1-S/F remodeled at the 10-g scale. The blue square is GlcNAc, the yellow filled circle is mannose, the green filled triangle is fucose, the red filled diamond is galactose, and the asterisk is sialic acid.

Table IV), indicating that *in vitro* glycosylation of sCR1 to yield sCR1-S or sCR1-S/F does not significantly impact the complement inhibitory properties of the molecule in the classical pathway.

In vitro *binding to E-selectin*

Figure 10 shows that sCR1-sLe^x and sCR1-S/F bind E-selectin in a concentration-dependent manner. The IC_{50}

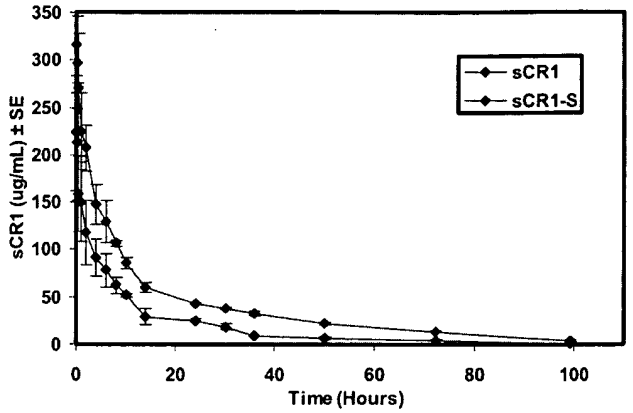


Fig. 8. The concentration of sCR1 and sCR1-S in plasma at various time points following bolus IV injection in rats.

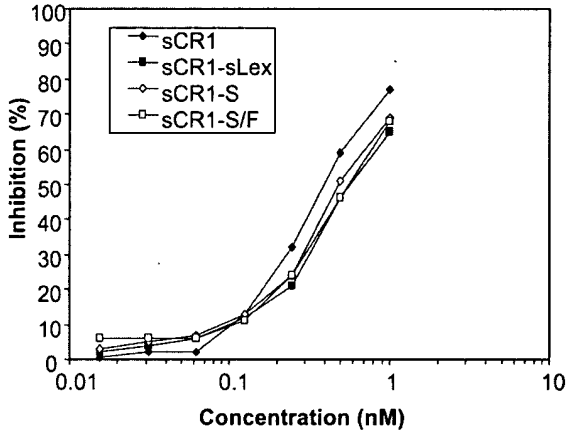


Fig. 9. Inhibition of red cell lysis via the classical pathway as a function of the concentration of sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F.

Table IV. Antihemolytic activity of modified sCR1 and sCR1-sLe^x

	IH_{50} (nM)
sCR1	0.41
sCR1-S	0.48 nM
sCR1-sLe^x	0.59
sCR1-S/F	0.59 nM

for sCR1-sLe^x from this plot is ~5 nM, and for sCR1-S/F ~0.4 nM. The observed 10-fold increase in inhibitory potency presumably is due to enhanced avidity, attributable to the increased density of sLe^x moieties on sCR1-S/F (28/mol) as compared with sCR1-sLe^x (14 per mol) (see Table I). The specificity of this binding was demonstrated by its calcium requirement and by the observation that sCR1

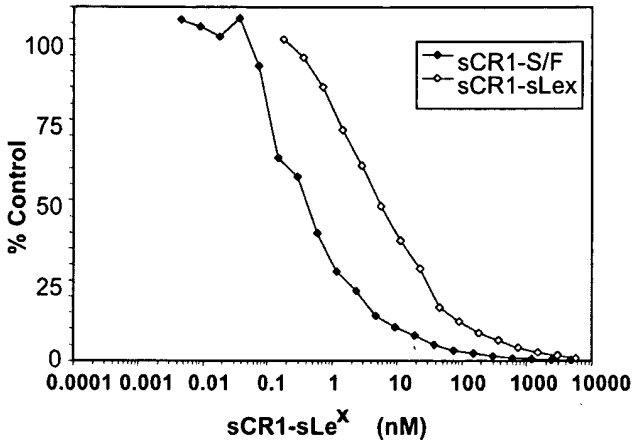


Fig. 10. Inhibition of PAA-sLe^x binding to E-selectin coated microtiter plates in the presence of varying concentrations of sCR1-sLe^x or sCR1-S/F.

(which does not contain any sLe^x structures) does not inhibit E-selectin binding at concentrations as high as 10 μM (data not shown).

Discussion

sCR1, made by standard CHO production methods, possesses predominantly biantennary oligosaccharides that are incompletely sialylated. We previously described an alternately glycosylated form of sCR1 called TP20 or sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), secreted by the FT-VI-expressing LEC11 CHO cell line and bearing sLe^x moieties on a fraction of its N-linked oligosaccharides. In this article we describe *in vitro* enzymatic remodeling of sCR1 by the stepwise application of two soluble recombinant glycosyltransferases in "one pot": The first step adds sialic acid to make sCR1-S, and the second adds fucose to make sCR1-S/F. The product of these glycan remodeling reactions contains an average of 28 sLe^x moieties per mol, as compared with 14 per mol found in CHO cell-produced sCR1-sLe^x.

That the sCR1 protein remains intact under conditions of glycan remodeling was demonstrated by RP-HPLC and SDS–PAGE analyses showing single polypeptides with expected molecular weights for sCR1-S and sCR1-S/F. Evidence for (1) conformational stability under conditions of the *in vitro* glycosylation reactions, and (2) preserved function despite variations in glycan structure, is provided by the observed near equivalence in bioactivity of sCR1, sCR1-S, sCR1-S/F, and CHO-produced sCR1-sLe^x in a standard complement inhibition assay.

The oligosaccharide structures associated with sCR1-S and sCR1-S/F were assessed by a number of methods. FACE profiling demonstrated a more fully sialylated set of glycoforms for sCR1-S as compared with sCR1 and nearly homogeneous, fully sialylated and fucosylated biantennary N-glycans for sCR1-S/F. Sequencing experiments using FACE provided supporting evidence that sialic acid

was linked α2-3 to galactose and that the predominant, single oligosaccharide band derived from sCR1-S/F was $BiNA_2F_2$. The analyses we performed do not establish linkages between the terminal and penultimate sugars that define sLe^x (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) versus sLe^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-). However, two factors make it likely that the glycans of sCR1-S/F do, in fact, terminate in sLe^x. First, it is known that in CHO cells, N-linked glycans are most commonly formed by β4GalT-1, and hence have the type-2 structure, Galβ1-4GlcNAcβ1- (Lee *et al.*, 2001). Second, the acceptor specificity of FT-VI is known to be restricted to type 2 chains (Costache *et al.*, 1997; Weston *et al.*, 1992).

During optimization of the sialylation reaction, we noted that incubation of sCR1 with either a low concentration of ST3Gal-III (10 mU/ml) for 24 h or a higher concentration (75 mU/ml) for 1 h produced a nearly maximally sialylated product. Even after incubation at the highest concentration of sialyltransferase tested (600 mU/ml for 24 h), a small fraction of monosialylated biantennary species persisted, perhaps due to steric hindrance at particular sites. Improved pharmacokinetics observed for the fully sialylated sCR1-S molecule as compared with sCR1 is probably a consequence of the added sialic acid blocking the interaction of terminal galactosyl residues with hepatic asialoglycoprotein receptors (Stockert, 1995).

We observed that FT-VI at 25 mU/ml fucosylates most sialylated biantennary glycans within 24 h. No significant differences were observed in catalytic activities of FT-VI expressed in the NSO cell line versus *Aspergillus niger* expression systems. The sCR1 polypeptide was shown to be stable following prolonged incubation with enzyme from either source.

In vitro glycosylation of sCR1 at the 10-g scale was carried out at enzyme concentrations selected to ensure nearly complete reaction at each stage. Success with the single experiment reported is consistent with the ability to predict useful scaled-up reaction conditions over a range of at least 40-fold based on mass of starting substrate. Both the ST3Gal-III and FT-VI enzymes used to glycosylate 10 g sCR1 were produced in *A. niger*, an expression system widely used for the manufacture of industrial enzymes in ton quantities. Although further scale-up would require refinement of incubation conditions, it can be estimated from present results that glycosylation of 1 kg of sCR1 might require 40,000 U ST3Gal-III and 20,000 U FT-VI, amounts that seem plausible to produce at reasonable cost in an industrial setting. To our knowledge, this is the largest scale reported enzymatic glycosylation of a glycoprotein to date by several orders of magnitude (Fischer and Dorner, 1998; Nemansky *et al.*, 1995; Paulson *et al.*, 1977; Raju *et al.*, 2001; Thotakura *et al.*, 1994).

The optimized conditions chosen for scale-up were very similar to the conditions used to generate material used for *in vivo* and *in vitro* studies. Compared with $sCR1\text{-}sLe^x$, sCR1-S/F was shown to have twice the number of sLe^x moieties and about a 10-fold higher apparent affinity for binding to E-selectin. This higher affinity presumably results from increased cooperativity in a multivalent binding reaction wherein sLe^x moieties distributed widely over sCR1-S/F engage multiple immobilized E-selectin molecules. In certain clinical situations, the anticomplement inhibitory and anti-inflammatory activity of sCR1-S/F could be effectively targeted via a similar mechanism to sites of inflammation where endothelial cells have been activated and have up-regulated expression of adhesion molecules including P- and E-selectin. $sCR1\text{-}sLe^x$ has been shown to be superior to sCR1 in a complement- and selectin-dependent lung injury model (Mulligan *et al.*, 1999), a murine model of ischemic stroke (Huang *et al.*, 1999), moderating skeletal muscle reperfusion injury (Kyriakides *et al.*, 2001a), moderation of acid aspiration injury (Kyriakides *et al.*, 2001b), reducing ischemia/reperfusion injury in rat lung grafts (Schmid *et al.*, 2001), and a myocardial ischemia and reperfusion model in the rat. $sCR1\text{-}sLe^x$ significantly reduced myocardial infarct size and was significantly more effective than sCR1 in reducing neutrophil infiltration into the infarction (Zacharowski *et al.*, 1999). It will be interesting to investigate whether sCR1-S/F is even more effective than $sCR1\text{-}sLe^x$ in similar animal models.

Materials and methods

Complement proteins, antibodies, enzymes, and other reagents

Purified sCR1 and $sCR1\text{-}sLe^x$ were prepared as previously described (Rittershaus *et al.*, 1999). Nucleotide sugars (CMP–sialic acid and GDP-fucose) were manufactured at Neose (Horsham, PA). CMP–sialic acid was prepared from CTP and sialic acid with recombinant CMP NeuAc synthetase (Shames *et al.*, 1991). GDP-fucose was either made from GDP-mannose using GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase/reductase, or purchased from Yamasa (Chiba, Japan). A gene encoding for a truncated, soluble form of ST3Gal-III (rat) was expressed in *A. niger* var. *awamori* dgr246 P2 using a variant of the expression vector pSL 1180 (Ward and Power, 2003). A 30–60% ammonium sulfate pellet was dissolved in 100 mM NaCl, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with 1 M NaCl, 20 mM MES, pH 6. rFT-VI (human) was expressed either in NSO cells or in *A. niger* as described as a soluble protein lacking the transmembrane domain. For the *A. niger* expressed protein, a 30–60% ammonium sulfate pellet was dissolved in 20 mM MES, pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM MES, pH 6. Soluble recombinant E-selectin was purchased from R&D Systems (Minneapolis, MN). Streptavidin–horseradish peroxidase conjugate (SA-HRP) was from Pierce (Rockford, IL), and biotinylated polyacrylamide polymer ($PAA\text{-}sLe^x$) was from GlycoTech (Rockville, MD). Anti-sCR1 monoclonal antibodies 6B1.H12 and 4D6.1 were prepared as previous described (Nickells *et al.*, 1998). Standards and glycosidases used in FACE analyses were from Glyko (Novato, California).

Preparation of sCR1-S

Lyophilized sCR1 (250 mg) was reconstituted and buffer exchanged into 50 mM Tris, 0.15 M NaCl, 0.05% NaN_3,

pH 7.2, using gel filtration columns (PD-10, Amersham Biosciences), and the concentration of sCR1 was adjusted to 5 mg/ml with the same buffer. Following addition of ST3Gal-III (150 mU/ml) and CMP–sialic acid (7 mM) the mixture was incubated at 32°C. A separate aliquot of the reaction mixture to which a trace amount of CMP–[^{14}C]sialic acid was added was incubated in parallel. From this, aliquot samples were withdrawn at various times and fractionated by isocratic HPLC/size-exclusion chromatography at 0.5 ml/min in 45% MeOH, 0.1% trifluoracetic acid (7.8 mm × 30 cm $TSKG2000_{SWXL}$ column, particle size 5 μm, TosoHaas). Incorporation of sialic acid into glycoprotein was calculated from the fraction of counts in the first eluted peak and the known concentration of sugar nucleotide.

Preparation of sCR1-S/F

After the sialylation reaction had proceeded for 48 h, GDP-fucose was added to a final concentration of 7 mM, $MnCl_2$ to 5 mM, and rFT-VI to 0.1 U/ml. A trace amount of GDP-[^{14}C]fucose was added to a separate aliquot, and both reaction mixtures were incubated at 32°C. Chromatography of the radiolabeled mixture as described showed the transfer of ~44 moles/mole sCR1-S after 48 h and 47 moles after 48 h. The product was provisionally designated sCR1-S/F.

Removal of nucleotide sugars and residual glycosyltransferases using ceramic hydroxyapatite and Q Sepharose chromatography

Glycosyltransferases and nucleotide sugars were removed from remodeled sCR1-S and sCR1-S/F by chromatography on ceramic hydroxyapatite (type I; BioRad, Hercules, CA) followed by Q Sepharose (Amersham Biosciences). Purity was assessed by RP-HPLC on a Poros R1/10 column (4.6 mmD/100 mmL, Applied Biosystems, Framingham, MA).

Optimization of sialylation and fucosylation reactions prior to scale-up

sCR1 was thawed slowly at 4°C and buffer exchanged into 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, using a PD10 column. *In vitro* sialylation of sCR1 (5 mg/ml) was evaluated using varying amounts of ST3Gal-III, 5 mM CMP–sialic acid, in the presence of 0.02% sodium azide at 32°C for 24 h. A trace amount of CMP–[^{14}C]sialic acid was added to an aliquot to monitor incorporation of radioactive sialic acid as described.

To the product (sCR1-S) of the reaction performed at a sialyltransferase concentration of 100 mU/ml (still containing the sialylation reagents) was added $MnCl_2$ and GDP-fucose, each to a final concentration of 5 mM, varing amounts of FT-VI, and a trace amount of GDP-[^{3}H]fucose. The resulting reaction mixture was incubated at 32°C for 24 h. Incorporation of radioactive fucose into the product (sCR1-S/F) was monitored as described for sialic acid.

sCR1 remodeling at 10-g scale

Purified sCR1 (10 g) was dialyzed exhaustively at 4°C against 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, adjusted to a concentration of 5 mg/ml with the same buffer, and incubated with ST3Gal-III (200 mU/ml) and CMP-sialic acid (5 mM) for 36 h at 32°C in a final volume of 2 L. After 36 h, an aliquot containing the sialylated product (sCR1-S) was withdrawn for analysis and the following reagents (final concentrations) were added: rFT-VI (100 mU/ml), GDP-fucose (5 mM), $MnCl_2$, (5 mM). After further incubation at 32°C for 36 h, a precipitate (manganese phosphate) was removed by centrifugation at 3000 × *g* for 5 min, and the sialylated and fucosylated product (sCR1-S/F) was stored at −70°C.

Monosaccharide analysis by HPLC

The neutral and amino sugar composition of glycoproteins was determined after trifluoroacetic acid hydrolysis and reductive amination with anthranilic acid by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1994). Sialic acid content was determined after sodium bisulfate hydrolysis and reaction with o-phenylenediamine by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1995).

Carbohydrate analysis by FACE

Carbohydrate sequencing and electrophoresis by FACE (Glyko and ProZyme, San Leandro, CA) was performed as previously described elsewhere (Picard *et al.*, 2000).

Carbohydrate analysis by 2-AA HPLC and MALDI-TOF MS

Glycans were released by PNGaseF and labeled with 2-AA according to the method described by Anumula and Dhume (1998) except that the labeled glycans were purified on cellulose cartridges (Glyko) according to the manufacturer's instructions. 2-AA-labeled N-glycans were analyzed using a Shodex Asahipak NH_2P-50 4D amino column (4.6 mm × 150 mm). The two solvents used for the separation were (A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and (B) 5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water. The column was eluted isocratically with 70% A for 2.5 min, followed by a linear gradient from 70% to 5% A over a period of 97.5 min, and a final isocratic elution with 5% A for 15 min. Eluted peaks were detected using fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 420 nm.

For MALDI-TOF analysis, a small aliquot of the 2-AA-labeled N-glycans was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 μm pore, 47 mm diameter) floating on water. The dialyzed aliquot was dried in a vacuum centrifuge, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water:acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Glycan structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully characterize isobaric structures.

SDS–PAGE

sCR1 samples before and after *in vitro* enzymatic remodeling were separated on 8–16% gradient Tris-glycine

polyacrylamide gels and stained with colloidal blue Coomassie stain. Gels, staining solutions, and molecular weight standards were obtained from Invitrogen (Carlsbad, CA).

Assays of complement regulatory activity

The inhibition of complement-mediated lysis of antibody-sensitized sheep erythrocytes (classical pathway) was assessed as previously described (Scesney *et al.*, 1996).

E-selectin binding assay

E-selectin binding assays were performed according to previously reported methods (Weitz-Schmidt *et al.*, 1996). Flat-bottom 96-well microtiter plates were coated with 5 μg/ml recombinant human E-selectin (R&D Systems) in 150 mM NaCl, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4 (HEPES-buffered saline, HBS). Coated wells were blocked with 2% bovine serum albumin/HBS. Varying concentrations of sCR1 or sCR1-sLe^x were added to the plate. A complex of a biotinylated polyacrylamide polymer containing sLe^x (PAA-sLe^x, GlycoTech) and SA-HRP was prepared. A dilution of this conjugate complex was added to the wells containing sCR1 or sCR1-sLe^x or buffer and incubated for 90 min at room temperature. The wells were washed with HBS/$CaCl_2$ and 3,3′,5,5′-tetramethylbenzidine substrate (KPL) was added to each well. Color was allowed to develop for 15 min, and the reaction was stopped with 2.0 N H_2SO_4. Bound PAA-sLe^x complex was measured by determining the absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Pharmacokinetic analysis in rats

Male Sprague-Dawley rats (~250 g), with in-dwelling jugular vein cannulas were purchased from Taconic (Germantown, NY) or Harlan Sprague Dawley (Indianapolis, IN). The catheters were periodically flushed with 0.9% saline followed by either heparinized glycerol (1:4 glycerol/333 IU heparin/ml) or heparinized saline (333 IU/ml) to ensure patency.

Animals were injected with sCR1 or sCR1-S (10 mg/kg) via the lateral tail vein as a bolus at time 0. Blood samples were obtained at timed intervals from the jugular vein cannula. The levels of sCR1 and sCR1-S present in the plasma samples were measured by a previously described enzyme-linked immunosorbent assay (Rittershaus *et al.*, 1999). Briefly, microtiter plates were coated with anti-sCR1 monoclonal antibody 6B1.H12 and captured sCR1 from a sample was detected with an HRP-conjugated anti-sCR1 monoclonal antibody 4D6.1. Pharmacokinetic data was analyzed using WinNonlin (Pharsight, Mountain View, CA).

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Abbreviations

2-AA, 2-anthranilic acid; AUC, area under the curve; CHO, Chinese hamster ovary; FACE, fluorophore-assisted carbohydrate electrophoresis; HBS, HEPES-buffered saline; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; RP-HPLC, reversed phase high-pressure liquid chromatography; SA-HRP, streptavidin–horseradish peroxidase; sCR1, soluble recombinant complement receptor type 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References

Anumula, K.R. (1994) Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. *Anal. Biochem.*, **220**, 275–283.

Anumula, K.R. (1995) Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal. Biochem.*, **230**, 24–30.

Anumula, K.R. and Dhume, S.T. (1998) High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology*, **8**, 685–694.

Costache, M., Apoil, P.A., Cailleau, A., Elmgren, A., Larson, G., Henry, S., Blancher, A., Iordachescu, D., Oriol, R., and Mollicone, R. (1997) Evolution of fucosyltransferase genes in vertebrates. *J. Biol. Chem.*, **272**, 29721–29728.

Fischer, B.E. and Dorner, F. (1998) Recombinant coagulation factor IX: glycosylation analysis and *in vitro* conversion into human-like sialylation pattern. *Thromb. Res.*, **89**, 147–150.

Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B., and Rasmussen, J.R. (1991) The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Biotechnology*, **9**, 1347–1355.

Huang, J., Kim, L.J., Mealey, R., Marsh, H.C. Jr., Zhang, Y., Tenner, A.J., Connolly, E.S. Jr., and Pinsky, D.J. (1999) Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. *Science*, **285**, 595–599.

Jenkins, N., Parekh, R.B., and James, D.C. (1996) Getting the glycosylation right: implications for the biotechnology industry. *Nat. Biotechnol.*, **14**, 975–981.

Kallio, E.A., Lemstrom, K.B., Hayry, P.J., Ryan, U.S., and Koskinen, P.K. (2000) Blockade of complement inhibits obliterative bronchiolitis in rat tracheal allografts. *Am. J. Respir. Crit. Care Med.*, **161**, 1332–1339.

Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001a) Moderation of skeletal muscle reperfusion injury by a sLe(x)-glycosylated complement inhibitory protein. *Am. J. Physiol. Cell Physiol.*, **281**, C224–C230.

Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001b) Sialyl Lewis(x) hybridized complement receptor type 1 moderates acid aspiration injury. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **281**, L1494–L1499.

Lasky, L.A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.*, **64**, 113–139.

Lazar, H.L., Bao, Y., Gaudiani, J., Rivers, S., and Marsh, H. (1999) Total complement inhibition: an effective strategy to limit ischemic injury during coronary revascularization on cardiopulmonary bypass. *Circulation*, **100**, 1438–1442.

Lee, J., Sundaram, S., Shaper, N.L., Raju, T.S., and Stanley, P. (2001) Chinese hamster ovary (CHO) cells may express six beta 4-galactosyltransferases (beta 4GalTs). Consequences of the loss of functional beta 4GalT-1, beta 4GalT-6, or both in CHO glycosylation mutants. *J. Biol. Chem.*, **276**, 13924–13934.

Lehmann, T.G., Koeppel, T.A., Kirschfink, M., Gebhard, M.M., Herfarth, C., Otto, G., and Post, S. (1998) Complement inhibition by soluble complement receptor type 1 improves microcirculation after rat liver transplantation. *Transplantation*, **66**, 717–722.

Mulligan, M.S., Warner, R.L., Rittershaus, C.W., Thomas, L.J., Ryan, U.S., Foreman, K.E., Crouch, L.D., Till, G.O., and Ward, P.A. (1999)

Endothelial targeting and enhanced antiinflammatory effects of complement inhibitors possessing sialyl Lewis x moieties. *J. Immunol.*, **162**, 4952–4959.

Naka, Y., Marsh, H.C., Seesney, S.M., Oz, M.C., and Pinsky, D.J. (1997) Complement activation as a cause for primary graft failure in an isogeneic rat model of hypothermic lung preservation and transplantation. *Transplantation*, **64**, 1248–1255.

Nemansky, M., De Leeuw, R., Wijnands, R.A., and Van Der Eijnden, D.H. (1995) Enzymic remodelling of the N- and O-linked carbohydrate chains of human chorionic gonadotropin. *Eur. J. Biochem.*, **227**, 880–888.

Nickells, M., Hauhart, R., Krych, M., Subramanian, V.B., Geoghegan-Barck, K., Marsh, H.C. Jr., and Atkinson, J.P. (1998) Mapping epitopes for 20 monoclonal antibodies to CR1. *Clin. Exp. Immunol.*, **112**, 27–33.

Paulson, J.C., Hill, R.L., Tanabe, T., and Ashwell, G. (1977) Reactivation of asialo-rabbit liver binding protein by resialylation with β-D-galactoside α2-6 sialyltransferase. *J. Biol. Chem.*, **252**, 8624–8628.

Picard, M.D., Pettey, C.L., Marsh, H.C., and Thomas, L.J. (2000) Characterization of N-linked oligosaccharides bearing sialyl Lewis x moieties on an alternatively glycosylated form of soluble complement receptor type 1 (sCR1). *Biotechnol. Appl. Biochem.*, **31**(1), 5–13.

Pratt, J.R., Hibbs, M.J., Laver, A.J., Smith, R.A., and Sacks, S.H. (1996) Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat. *Am. J. Pathol.*, **149**, 2055–2066.

Pruitt, S.K., Bollinger, R.R., Collins, B.H., Marsh, H.C., Levin, J.L., Rudolph, A.R., Baldwin, W.M., and Sanfilippo, F. (1997) Effect of continuous complement inhibition using soluble complement receptor type 1 on survival of pig-to-primate cardiac xenografts. *Transplantation*, **63**, 900–902.

Raju, T.S., Briggs, J.B., Chamow, S.M., Winkler, M.E., and Jones, A.J.S. (2001) Glycoengineering of therapeutic glycoproteins: *in vitro* galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. *Biochemistry*, **40**, 8868–8876.

Rittershaus, C.W., Thomas, L.J., Miller, D.P., Picard, M.D., Geoghegan-Barck, K.M., Seesney, S.M., Henry, L.D., Sen, A.C., Bertino, A.M., Hannig, G., and others. (1999) Recombinant glycoproteins that inhibit complement activation and also bind the selectin adhesion molecules. *J. Biol. Chem.*, **274**, 11237–11244.

Seesney, S.M., Makrides, S.C., Gosselin, M.L., Ford, P.L., Andrews, B.M., Hayman, E.G., and Marsh, H.C. (1996) A soluble deletion mutant of the human complement receptor type 1, which lacks the C4b binding site, is a selective inhibitor of the alternative complement pathway. *Eur. J. Immunol.*, **26**, 1729–1735.

Schmid, R.A., Hillinger, S., Hamacher, J., and Stammberger, U. (2001) TP20 is superior to TP10 in reducing ischemia/reperfusion injury in rat lung grafts. *Transplant. Proc.*, **33**, 948–949.

Shames, S.L., Simon, E.S., Christopher, C.W., Schmid, W., Whitesides, G.M. and Yang, L.L. (1991) CMP-N-acetylneuraminic acid synthetase of *Escherichia coli*: high level expression, purification and use in the enzymatic synthesis of CMP-N-acetylneuraminic acid and CMP-neuraminic acid derivatives. *Glycobiology*, **1**, 187–191.

Stammberger, U., Hamacher, J., Hillinger, S., and Schmid, R.A. (2000) sCR1sLe^x ameliorates ischemia/reperfusion injury in experimental lung transplantation. *J. Thorac. Cardiovasc. Surg.*, **120**, 1078–1084.

Stockert, R.J. (1995) The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol. Rev.*, **75**, 591–609.

Thotakura, N.R., Szkudlinski, M.W., and Weintraub, B.D. (1994) Structure-function studies of oligosaccharides of recombinant human thyroptrophin by sequential deglycosylation and resialylation. *Glycobiology*, **4**, 525–533.

Ward, M. and Power, S.D. (2003) DNA sequences, vectors, and fusion polypeptides for secretion of polypeptides in filamentous fungi. US Patent no. 6,590,078 B2.

Weisman, H.F., Bartow, T., Leppo, M.K., Marsh, H.C., Carson, G.R., Concino, M.F., Boyle, M.P., Roux, K.H., Weisfeldt, M.L., and Fearon, D.T. (1990) Soluble human complement receptor type 1: *in vivo* inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science*, **249**, 146–151.

Weitz-Schmidt, G., Stokmaier, D., Scheel, G., Nifant'ev, N.E., Tuzikov, A.B., and Bovin, N.V. (1996) An E-selectin binding assay based on a polyacrylamide-type glycoconjugate. *Anal. Biochem.*, **238**, 184–190.

Weston, B.W., Smith, P.L., Kelly, R.J., and Lowe, J.B. (1992) Molecular cloning of a fourth member of a human α(1,3)fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis x, sialyl Lewis x, and difucosyl sialyl Lewis x epitopes. *J. Biol. Chem.*, **267**, 24575–24584.

Williams, J.P., Pechet, T.T., Weiser, M.R., Reid, R., Kobzik, L., Moore, F.D. Jr., Carroll, M.C., and Hechtman, H.B. (1999) Intestinal reperfusion injury is mediated by IgM and complement. *J. Appl. Physiol.*, **86**, 938–942.

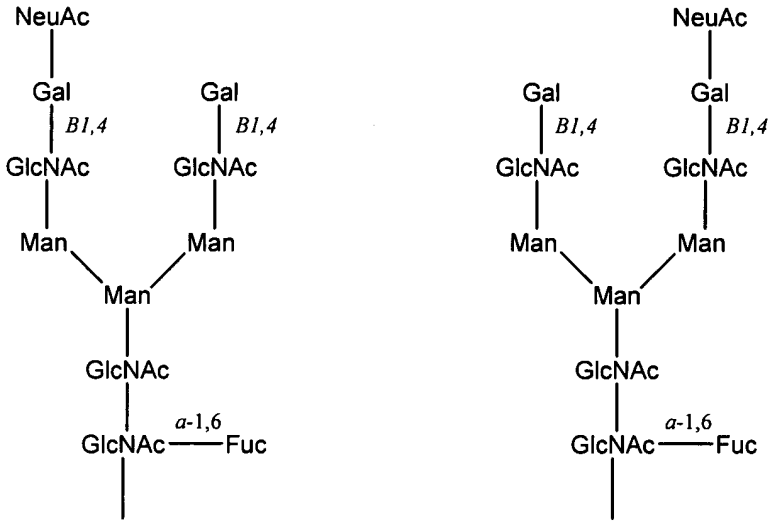
Zacharowski, K., Otto, M., Hafner, G., Marsh, H.C. Jr., and Thiemermann, C. (1999) Reduction of myocardial infarct size with sCR1sLe(x), an alternatively glycosylated form of human soluble complement receptor type 1 (sCR1), possessing sialyl Lewis x. *Br. J. Pharmacol.*, **128**, 945–952.

Zhang, A., Potvin, B., Zaiman, A., Chen, W., Kumar, R., Phillips, L., and Stanley, P. (1999) The gain-of-function Chinese hamster ovary mutant LEC11B expresses one of two Chinese hamster FUT6 genes due to the loss of a negative regulatory factor. *J. Biol. Chem.*, **274**, 10439–10450.

Exhibit 2C

N-linked glycan structures

A.



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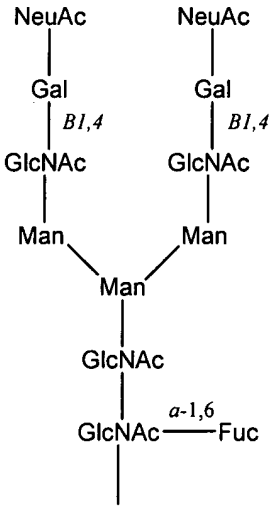

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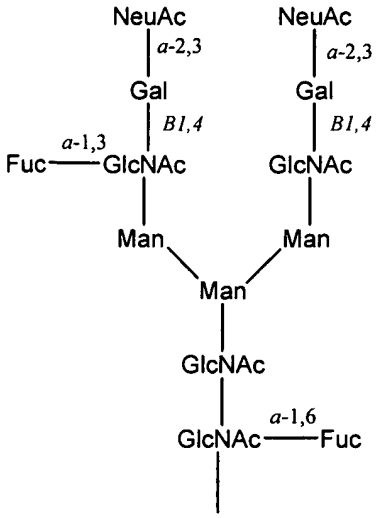

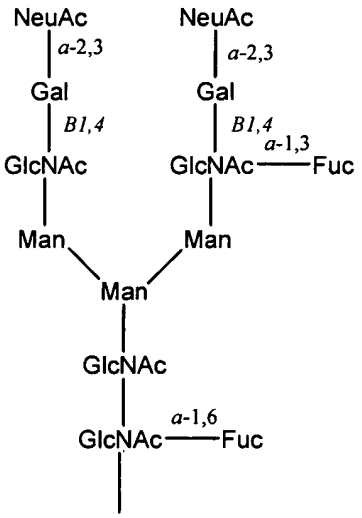

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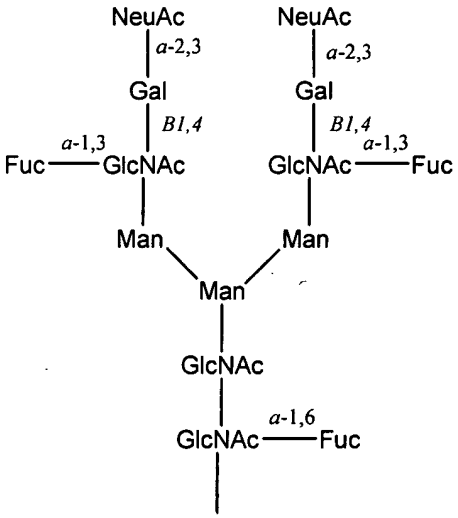
D.



A. Monosialylated, biantennary core-fucosylated glycan

B. Disialylated, biantennary core-fucosylated glycan

C. Monofucosylated, disialylated, biantennary core-fucosylated glycan

D. Difucosylated, disialylated, biantennary core-fucosylated glycan

Exhibit 2D

Fucosylation Conditions

14:1 Donor : Acceptor ***Example 1,'806 Application***	***7:1 Donor : Acceptor*** ***Thomas***
2.5 mg/mL sCR1-S	5 mg/mL sCR1-S
5 mM GDP-fucose	5 mM GDP-fucose
0.05 U/mL FT-VI	0.1 U/mL FT-VI
32 °C for 2 days	32 °C for 36 hours